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Tyrosine Aminotransferase Induction in Hepatocytes Cultured from Rat Foetuses Treated with Dexamethasone *in utero*

By GEORGE C. T. YEOH,* TYE ARBUCKLE* and IVAN T. OLIVER†
Departments of Physiology and Biochemistry,† University of Western Australia, Nedlands, Perth 6009, Australia*

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1. The administration of dexamethasone to foetal rats *in utero* does not result in the appearance of specific tyrosine aminotransferase activity even after 24h. 2. When foetal hepatocytes are cultured *in vitro* from animals treated *in utero* with dexamethasone, significantly higher activities of specific tyrosine aminotransferase are found than in untreated controls. 3. Dexamethasone *in vitro* induces specific tyrosine aminotransferase in cells cultured from control animals, and the effect is maximal at 10nM in the culture medium. 4. Actinomycin D at 0.2μg/ml in the culture medium completely prevents the induction of activity *in vitro*. 5. In cultures established from animals treated with dexamethasone *in utero*, the increase in specific tyrosine aminotransferase activity over the control cultures is only marginally decreased in the presence of actinomycin D. 6. The results can be interpreted to mean that dexamethasone *in utero* stimulates the transcription of enzyme-specific mRNA, which is not translated until a translational block in the foetal liver is removed by the conditions of culture *in vitro*.

The liver enzyme tyrosine aminotransferase (EC 2.6.1.5) is absent from foetal rat liver and cannot be precociously induced *in utero* by steroid administration (Sereni *et al.*, 1959; Greengard, 1969). Significant amounts of enzyme accumulate within a few hours of birth. However, the liver will synthesize the enzyme if the foetus is delivered prematurely (Holt & Oliver, 1968) or if the liver is cultured, either as an explant (Wicks, 1969) or as a monolayer of cells (Yeoh *et al.*, 1977). The administration of corticosteroid to the prematurely delivered foetus (Greengard & Dewey, 1967; Cake *et al.*, 1973) or the addition of steroid or steroid analogue to the culture medium (Yeoh *et al.*, 1977) markedly enhances the activity of tyrosine aminotransferase.

Several explanations have been put forward to account for the failure of enzyme induction by steroid in the foetus. The hormone may require to be converted into an active product, but the foetal liver may lack the metabolic mechanism. However, the studies of Cake (1974) suggest that this is unlikely. Alternatively the steroid-receptor protein, which acts as an intracellular carrier for the steroid (Cake & Litwack, 1975), or some other component of the pathway involved with the nuclear interactions of the steroid (Baxter *et al.*, 1972; Cake & Litwack, 1975) may be absent from the foetal liver. These possibilities were investigated by Feldman (1974) and Giannopoulos (1975). It was concluded that the lack of steroid inducibility of liver tyrosine aminotransferase in the foetus was not due to a lack of steroid receptor or nuclear interactions.

This communication presents and discusses results obtained from a series of experiments in which rat foetuses were given steroid *in utero* and their livers subsequently cultured. The tyrosine aminotransferase activity in these cultured hepatocytes suggests that the steroid has effectively modified a step or steps in the sequence of events which culminates in the production of the enzyme. In addition, the enzyme yield in cultures maintained continuously in actinomycin D suggests that the steroid affects transcription of the tyrosine aminotransferase gene when administered *in utero*.

Materials and Methods

Materials

Animals. Wistar albino rats were used. The animals have a gestation period of 22 days. Gestational age was determined from the time of detection of spermatozoa in the vaginal tract of females and is accurate to $\pm$8h.

Chemicals. L-[3,5-^{3}H]Tyrosine, [5-^{3}H]uridine, [1-^{14}C]leucine and [1,2-^{3}H]dexamethasone were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. L-Tyrosine and sodium diethyldithiocarbamate were from Merck, Darmstadt, Germany. Pyridoxal 5-phosphate, 2-oxoglutarate, dexamethasone and actinomycin D were purchased

from Sigma Chemical Co., St. Louis, MO, U.S.A. Collagenase (grade II), cycloheximide, cordycepin and puromycin came from Boehringer/Mannheim, Mt. Waverley, Vic., Australia. 2,4-Dinitrophenylhydrazine and L-aspartate were from Ajax Chemicals, Sydney, Australia; hydroxyapatite was supplied by Bio-Rad Laboratories, Richmond, CA, U.S.A., CM-Sephadex (C-50) by Pharmacia Fine Chemicals, Uppsala, Sweden, and diaminobenzoic acid by the Eastman–Kodak Co., Rochester, NY, U.S.A. Cell-culture reagents, Fungizone (amphoterecine B), penicillin/streptomycin and glutamine were obtained from Grand Island Biological Co., Grand Island, NY, U.S.A. Eagle's minimal essential medium and foetal calf serum were from Flow Laboratories, Annandale, N.S.W., Australia.

Methods

Injection of animals. Foetal animals were injected *in utero* as described by Yeung *et al.* (1967). Control foetuses (20 μl of propylene glycol) or test foetuses (64 μg of dexamethasone in 20 μl of propylene glycol) were injected by using a 30-gauge dental needle attached by cannula tubing to an Agla syringe driven by a micrometer (Burroughs Wellcome, Beckenham, Kent, U.K.). In each pregnant rat, the foetuses in one uterine horn were used as controls and in the other uterine horn as tests. Controls and tests from two litters were pooled and hepatocytes from each group cultured in 90mm-diameter culture dishes. Foetuses were exposed to dexamethasone *in utero* for 4h.

Hepatocyte isolation. Foetal rats were delivered by Caesarian section, livers were removed aseptically and rinsed free of blood in balanced salts solution (Hanks & Wallace, 1949) and dried on sterile paper napkins. Livers were chopped on a Mickle Chopper (The Mickle Laboratories Engineering Co., Gomshall, Surrey, U.K.) set at 0.1mm using two traverses, one perpendicular to the other. The pieces were then placed in balanced salts solution containing 0.5mg of collagenase/ml in a flask. About 500mg of liver/ml of collagenase solution was used. The flask was then gassed with carbogen (O_2/CO_2, 19:1) and incubated with shaking at 37°C for 15min. An equal volume of balanced salts solution was then added to this mixture, which was filtered through cheesecloth. The cells were pelleted and washed twice in balanced salts solution by centrifugation at 50*g* for 2min. Finally the cells were suspended (approx. 0.5ml of packed cells/10ml of medium) in modified Eagle's minimal essential medium supplemented with 10% foetal calf serum, glutamine (2.4mM final concentration), Fungizone (28 μg/ml) and penicillin/streptomycin (57units/ml, 570 μg/ml). Cells were then plated in 90mm-diameter plastic culture dishes (Sterillin products, Teddington, Middx., U.K.), previously coated with collagen in a volume of 9ml.

Hepatocyte cultures. Cultures were given fresh medium 24h after plating and every 48h thereafter. The initial removal of medium results in the removal of most of the erythropoietic cells, which do not attach to the substrate. Cultures were maintained at 37°C in a water-saturated atmosphere of 5% CO_2 in air.

Preparation of extracts from hepatocyte cultures. Cells were harvested by using a Teflon 'policeman' and suspended in balanced salts solution. A cellular pellet was obtained by centrifugation at 1600*g* for 2min in a Clements GS 100 bench centrifuge. The pellet was resuspended in 0.05M-potassium phosphate buffer, pH6.5, and sonicated for 15s at 2A d.c. in a Branson model L5 75 sonifier. The sonicated liver was incubated for 45min in an ice bath to ensure conversion of multiple forms of the enzyme into a single form (Smith *et al.*, 1975). Samples of 100–200 μl were centrifuged in a Beckman Airfuge (Beckman Instruments, Palo Alto, CA, U.S.A.) at 207kPA for 12min in the A-100 rotor. Centrifugation was performed at 4°C.

Removal of aspartate aminotransferase from extracts. This was accomplished by chromatography on CM Sephadex C-50 (Pharmacia Fine Chemicals), at 4°C. Extracts (100–200 μl) were run into columns (0.7cm × 3cm) washed with 1ml of 0.06M-potassium phosphate buffer, pH6.5, then left at 4°C for 45min. Aspartate aminotransferase was removed by elution with 5ml of 0.05M-KCl in 0.05M-potassium phosphate buffer, pH6.5. Tyrosine aminotransferase was then eluted with sequential 1ml portions of 0.33M-KCl in 0.05M-potassium phosphate, pH6.5. Most of the activity appeared in the second eluate.

Tyrosine aminotransferase assay. The enzyme was determined by a modification of the methods of Miller & Thompson (1972) and Lees & Weiner (1975). The initial mixture contained 2-oxoglutarate (5mM), pyridoxal 5-phosphate (0.13mM), diethyldithiocarbamate (53mM) and L-tyrosine (3mM), in 0.1M-potassium phosphate buffer, pH7.4. L-[3,5-^{3}H]-Tyrosine (approx. 1 μCi) was added to 1ml of this mixture. A sample (100 μl) was counted for radioactivity in 10ml of a scintillant solution consisting of naphthalene (180g), 2,5-diphenyloxazole (4g) and dioxan (1 litre).

The reaction mixture (100 μl) was preincubated at 37°C in a shaking water bath and the reaction started by addition of enzyme. 2,4-Dinitrophenylhydrazine (15mM) in H_2SO_4 (1M) was used to stop the reaction. Blanks were prepared by addition of 2,5-dinitrophenylhydrazine before adding the enzyme. The product was extracted into 1ml of toluene/ethyl acetate (10:1, v/v) and the solution centrifuged at 1600*g* for 15min. The organic phase (0.7ml) was added to 0.3M-HCl (0.5ml), mixed and centrifuged

as above. The radioactivity in the organic phase was determined in an Isocap 300 liquid-scintillation counter (Nuclear–Chicago) in a scintillant containing 2,5-diphenyloxazole (6g) and ethanol (0.5ml) per litre of toluene. The counting efficiency was 40%. Tyrosine aminotransferase activity was calculated as nmol of *p*-hydroxyphenylpyruvate/h per mg of DNA when assayed in total supernatants and nmol of *p*-hydroxyphenylpyruvate/h per mg of protein in aspartate aminotransferase-free supernatants.

RNA and protein synthesis. RNA synthesis was measured by the incorporation of [^{3}H]uridine introduced into the culture (0.5μCi/ml) at the time of plating. After 18h, the cells were harvested, washed by centrifugation and sonicated (2A d.c. 15s in a Branson model L5 75 sonifier). Total nucleic acids were extracted into hot trichloroacetic acid (0.3M) at 90°C for 30min and a sample was taken for radioactivity determination by liquid-scintillation counting. Protein synthesis was measured by the incorporation of [^{14}C]leucine (0.5μCi/ml of medium) into material precipitated by 0.3M-trichloroacetic acid. The precipitate was dissolved in 0.1M-NaOH, of which samples were taken for radioactivity determination.

Control experiments. To check the transference of dexamethasone from test animals to control animals and from test animals into the culture medium, foetuses were injected with 1μCi of [^{3}H]dexamethasone together with the normal dose. Livers were then removed at various times from controls and tests and digested overnight in NCS (Nuclear–Chicago) tissue solubilizer before samples were taken for determination of radioactivity. Other test livers were prepared for culture, except that the final suspension of cells was centrifuged, dissolved in NCS and samples were taken for counting.

Protein was determined by the method of Lowry *et al.* (1951), with a bovine serum albumin standard. DNA was determined by the fluorescence method of Hinegardner (1971) by using highly polymerized calf thymus DNA (Sigma Chemical Co.) as a standard.

The paired difference *t* test was used to determine statistical significance.

Results

The dose of dexamethasone (64μg/foetus) given *in utero* has previously been shown to be effective in inducing tyrosine aminotransferase in postnatal and prematurely delivered rats (Cake *et al.*, 1973).

However, after a 4h exposure to this dose of steroid *in utero*, tyrosine-transaminating activity of livers of 19-day foetal rats was 174 ± 11 compared with 169 ± 3 units in control livers. When aspartate aminotransferase was removed from the extracts by chromatography on CM-Sephadex C50 before assay, no tyrosine transamination was detected in fresh liver extracts from either group of foetuses. Similar results were obtained when the exposure to dexamethasone was extended to 24h. When hepatocytes from livers exposed to steroid *in utero* for 4h were then cultured for 18h, the tyrosine aminotransferase activity of steroid-treated cells reached 158 ± 16, whereas that of control cells was only 63 ± 9 units ($P < 0.001$). The enzyme activity has been shown to vary with different batches of foetal calf serum, age of the culture medium and the batch of collagenase used to disperse the cells. The effect of foetal calf serum is presumably due to variation in endogenous steroid, the presence of which was verified by assay. However, reproducibility of the enzyme activity within a single lot of cultured cells was $\pm 15\%$.

The sensitivity of 18h-cultured hepatocytes (derived from 19-day gestation rats) to dexamethasone added to the culture medium is shown in Table 1. A marginal effect is obtained at concen-

Table 1. *Tyrosine aminotransferase activity in cultured hepatocytes as a function of dexamethasone concentration*
Hepatocytes from 19-day-gestation rats were cultured as described in the Materials and Methods section. The medium was supplemented with the indicated concentrations of dexamethasone from the time of plating. After 18h cells were collected and assayed for tyrosine aminotransferase as described in the Materials and Methods section. Two 90mm dishes of cells at each dexamethasone concentration were assayed. The means of results that agreed within 10% are presented.

Dexamethasone concentration (M)	Tyrosine aminotransferase activity (nmol/h per mg of protein)	Percentage increase
None	21	
10^{-11}	23	10
10^{-10}	28	30
5×10^{-10}	30	40
10^{-9}	57	70
10^{-8}	200	850
10^{-7}	177	740
10^{-6}	156	640

trations below 1 nM, whereas maximal induction is achieved with 10 nM-dexamethasone.

In order to determine the fate of administered steroid, a tracer amount of [^{3}H]dexamethasone was given together with the standard dose. Maximum accumulation by the liver of dexamethasone from a dose of this amount is achieved within 5 min of injection. This represents about 13% of the dose and rapidly falls to around 1.5% after 1 h. At this time, in littermates that did not receive dexamethasone but an equal volume of the vehicle propylene glycol, transfer was maximal and amounted to 2% of the concentration in test animals (0.3% of total dose). Finally the radioactivity recovered in the cells prepared from the livers of dexamethasone-treated animals represents 0.003% of the total dose.

Table 2 summarizes the result of experiments designed to select the most appropriate transcriptional and translational inhibitors and the optimal concentrations for use with cultured hepatocytes. The data indicate that the translational inhibitors puromycin and cycloheximide, at dosages that gave 72 and 86% inhibition of protein synthesis respectively, also decreased RNA synthesis too much to be used. However, actinomycin D at a dose of 0.2 μg/ml markedly diminished RNA synthesis (88%), whereas protein synthesis was decreased by only 13% under the culture conditions. This inhibitor was selected in preference to cordycepin.

When actinomycin D (0.2 μg/ml) was present in the culture medium, induction of tyrosine aminotransferase by dexamethasone (10 nM) was significantly decreased. Activity was increased by 300% in controls, whereas a marginal increase of 22% was obtained when RNA synthesis is blocked (Table 3).

Table 2. *Effect of cycloheximide, puromycin, actinomycin D and cordycepin on RNA and protein synthesis in cultured hepatocytes*

Cultures were set up as described in the Materials and Methods section. They were exposed to cycloheximide (1.3 μg/ml), puromycin (10 μg/ml), actinomycin D (0.2 μg/ml) or cordycepin (28 μg/ml). RNA synthesis was measured by the incorporation of [^{3}H]uridine into RNA and protein synthesis was measured by the incorporation of [^{14}C]leucine into trichloroacetic acid-insoluble material. The means of results from two experiments, each using three culture dishes per test, are presented.

	Percentage inhibition	
	RNA synthesis	Protein synthesis
Cycloheximide	50	86
Puromycin	74	72
Actinomycin D	88	13
Cordycepin	73	15

Table 3. *Effect of actinomycin D on the dexamethasone induction of tyrosine aminotransferase in cultured hepatocytes*

Hepatocytes were cultured without additives (control), with dexamethasone (10 nM), with actinomycin D (0.2 μg/ml), or with actinomycin and dexamethasone. Tyrosine aminotransferase activity was determined after 18 h of culture as described in the Materials and Methods section. For each group of data three cultures were set up and the means ± S.E.M. of results are presented.

	Tyrosine aminotransferase activity (nmol/h per mg of protein)
Control	66 ± 4.7
Dexamethasone	266 ± 42.6
Actinomycin D	51 ± 6.8
Actinomycin D and dexamethasone	62 ± 4.4

In spite of transcription being blocked by actinomycin D during 18 h of culture, hepatocytes from 19-day control foetuses still accumulated 36 ± 5 units of tyrosine aminotransferase activity. Furthermore, significantly higher enzyme activity (76 ± 11 units, $0.01 > P > 0.001$) was present in hepatocytes cultured from foetuses exposed to dexamethasone *in utero*.

Discussion

When dexamethasone is given to foetal rats *in utero* it fails to induce synthesis of tyrosine aminotransferase in the liver. Although the sensitive radiochemical assay detects significant transamination of tyrosine of about the same activity in controls and test animals, it has been demonstrated that this can be ascribed to the non-specific activity of aspartate aminotransferase (Yeoh *et al.*, 1979). This is consistent with the findings of Greengard & Dewey (1967) and Cake *et al.* (1973). When this enzyme is removed by chromatography on CM-Sephadex, no detectable activity is obtained in either controls or test animals. However, when hepatocytes prepared from these livers are cultured for 18 h tyrosine aminotransferase accumulates. This does not occur *in utero*, even at 24 h after dexamethasone administration, and cannot therefore be explained as an age-related phenomenon. Of special significance is the finding that more enzyme (2.5 times) is detected in cells obtained from foetuses that received dexamethasone *in utero*. This suggests that the steroid has had some effect *in utero*, unless the observed phenomenon is an artifact due to the transfer of the injected steroid to the culture. This possibility has to be considered in view of the sensitivity of cultured hepatocytes to dexamethasone added to the culture medium. The use of [^{3}H]dexamethasone as a tracer reveals that 0.003% of the administered steroid is

transferred into the culture. Assuming that this is evenly distributed in the culture and 100% active, it represents 0.5 nM-dexamethasone, a concentration that increases enzyme activity by only 40% compared with the 251% increase in the experiments where dexamethasone is given *in utero*. When the experiment is repeated with cultures set up in actinomycin D, the data reveal that tyrosine aminotransferase still accumulates and that those hepatocytes isolated from foetuses that had received dexamethasone contain significantly more tyrosine aminotransferase.

The results lead us to conclude that in 19-day-gestation foetal liver, although tyrosine aminotransferase is undetectable, mRNA coding for the enzyme may be already present. This mRNA could be transcribed as a result of the high concentrations of plasma corticosterone in the foetus at this stage of development (Martin *et al.*, 1977) and this is suggested by the appearance of enzyme activity in cultures derived from uninjected foetal litters maintained in actinomycin D (Table 3). In the hormone experiments transcription might occur *in utero* as a result of dexamethasone transfer from injected littermates to the controls. Whatever the source of steroid, it is proposed that in the environment *in utero* the message is not translated. The appearance of the enzyme within 18 h of culture suggests that a translational block is released when the hepatocytes are placed in culture medium. Since cultured hepatocytes translate more tyrosine aminotransferase when they have been exposed to exogenous steroid *in utero*, it is reasonable to suggest that the steroid has probably increased the amount of mRNA coding for the enzyme in the foetus. This can only be unequivocally demonstrated by direct assay of the message in foetal liver after steroid administration. An increase in the amount of translatable mRNA for tyrosine aminotransferase as a result of steroid administration to adult rats has been reported (Diesterhaft *et al.*, 1977). Some of the data presented here indicate that enzyme induction *in vitro* is also dependent upon RNA synthesis, for it is blocked by the presence of actinomycin D.

In summary, the data presented indicate that steroids manifest activity when administered *in utero* and are consistent with the proposition that dexamethasone stimulates the transcription of enzyme-specific mRNA. A response with respect to induced enzyme production is not observed *in utero* because of a block in translation, the nature of which deserves investigation.

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