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EFFECT OF CULTURE AGE ON DRUG METABOLIZING ENZYMES AND THEIR INDUCTION IN PRIMARY CULTURES OF RAT HEPATOCYTES

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Abstract—In order to further establish optimal and reproducible conditions for the use of primary hepatocyte cultures in studies of drug metabolism, the effect of culture age on the basal and induced activities of ethoxycoumarin *O*-deethylase (ECDE), UDP-glucuronyltransferase (GT) [methylumbelliferone (MU) and *p*-nitrophenol (pNP) as substrates] and sulfotransferase (MU) were measured. In contrast to the monooxygenase activity conjugating activities were maintained for 2–3 weeks in culture, although especially sulfate conjugation showed a transient decline during the first days, and GT activity increased later on during culture. Low induction of ECDE with both phenobarbital (PB) and 3-methylcholanthrene (MC) was seen during the first day in culture, and maximum induction was obtained when inducer was added on the second or third day. The MC inducible GT (pNP) exhibited a similar behaviour indicating that the coordinated induction of the MC inducible activities is preserved in culture. The results show that primary cultures of hepatocytes can be used to study conjugating enzymes and their regulation. However, each functional parameter that is to be investigated in hepatocyte cultures should first be studied as a function of culture age to establish the optimum time.

Studies on drug metabolism in cultured hepatocytes have focused on the mixed function oxidases, where, however, the rapid loss of cytochrome P-450 in culture is a major problem. To prevent this loss modifications of the culture medium, such as addition of hormones [1], nicotinamide or other pyridines [2], or omission of certain amino acids [3] have been tried, but none of these have succeeded to more than partly prevent the decline, and changes in the relative amounts of the cytochrome P-450 isozymes may still be taking place [4].

In contrast, the behaviour of the conjugating enzymes in cultured hepatocytes has been very little studied. However, when considering the potential of cultured hepatocytes for drug metabolism studies a knowledge of the overall metabolism is essential, since conjugation reactions are of importance, e.g. in removing reactive metabolites formed by the mixed-function oxidases. Since conjugating enzymes are inducible as well, the usefulness of hepatocyte cultures for the investigation of their regulation should also be considered.

The present study was undertaken to study the relationship between conjugation reactions and monooxygenase reactions in both uninduced and induced cultures. Since it is evident that on one hand the isolation process causes damage to hepatocytes, from which they at least partly recover in culture, but on the other hand the so far still imperfect culture conditions also cause a deterioration of the cells, this work also tried to establish an optimum culture age for measuring drug metabolizing enzymes and their induction.

MATERIALS AND METHODS

Cell isolation and culture. Hepatocytes were isolated from male Sprague–Dawley rats with the collagenase perfusion method of Seglen [5] under aseptic conditions. Viability was determined by trypan blue exclusion, viabilities were around 90%. The cells were suspended in growth medium at 0.6–0.7 × 10^6 viable cells/ml and 2.5 ml or 6.5 ml of the suspension was inoculated into 50 mm or 85 mm Petri dishes, respectively. The dishes had been coated with a collagen (rat tail) gel according to Michalopoulus and Pitot [6]. The growth medium was a 1:1 mixture of Waymouth MB 752/1 and Ham F-12, supplemented with 20 mmol/l Hepes, 5 mmol/l $NaHCO_2$, 10 mmol/l Tricine, 10 μg/ml gentamycin, 10 IU/l insulin, 50 μg/l glucagon, 10^{-6} mol/l dexamethasone, 5% fetal calf serum and 5% newborn calf serum. To some cultures metyrapone* (MP) (2-methyl-1,2di-3-pyridyl-1-propanone) was added at 0.5 mmol/l; in induction studies either PB at a final concentration of 2 mmol/l or MC at 5 μmol/l were used.

Enzyme assays. Conjugation of MU was measured in whole cells, attached to the collagen gel. The dishes were washed with phosphate buffered saline and then incubated for 1 hr at 37° in the presence of 125 μmol/l substrate and 50 mmol/l sulfate. MUG and MUS were quantitated after removal of unreacted substrate and hydrolysis with β-glucuronidase and HCl, respectively, as described earlier [7]. For other determinations the cells were detached with collagenase (1 mg/ml PBS), washed with PBS, and stored frozen until homogenization in 0.15 mol/l KCl. ECDE was determined according to Aitio [8],

* Abbreviations used: MU, methylumbelliferone; MUG, methylumbelliferone glucuronide; MUS, methylumbelliferone sulfate; pNP, *p*-nitrophenol; MC, 3-methylcholanthrene; PB, phenobarbital; GT, UDP-glucuronyltransferase; MP, metyrapone; ECDE, 7-ethoxycoumarin *O*-deethylase; PBS, phosphate buffered saline.

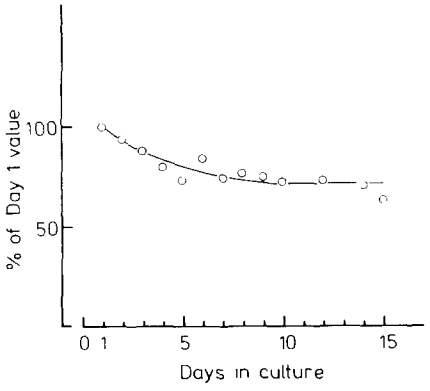

Fig 1 The DNA content of hepatocyte cultures as a function of culture time The 100% value represents the cells that have attached to collagen gels after the first medium change (at 4 hr) The values given are means from 3–10 separate experiments (2 dishes/point in each experiment) The S E between different experiments ranged from 1 5 to 5 6% For details see Materials and Methods

and pNP glucuronidation in the absence of detergent according to Bock *et al* [9] DNA was determined according to Richards [10] For freshly isolated cells the DNA content was 14 2 $\mu g/10^6$ cells.

Chemicals Collagenase (Type IV), β-glucuronidase (Type B-1), insulin (bovine pancreas), dexamethasone, glucagon, metyrapone, ethoxycoumarin, methylumbelliferone, NADP, UDP-glucuronic acid and buffer materials were from Sigma Chemical Co (St. Louis, MO), and the media and sera from GIBCO (Paisley, Scotland)

RESULTS

The DNA content as a function of culture time is shown in Fig. 1 There is an initial loss during the first five days, after which the DNA content stabilizes at about 75% of the day-1 level This DNA level is retained for at least 2 weeks—some cultures have been kept for up to 3 weeks. There is some variation between cultures, which does not seem to be related to the original viability or other known factors Occasionally there was a culture where the DNA content decreased very rapidly (these have not been included in Fig 1)

Drug metabolism in cultured cells

Uninduced cultures In freshly isolated cells (measured from whole cells) the activity of GT with MU as substrate was 23 5 pmol/min/μg DNA and the sulfotransferase activity 7.2 pmol/min/μg DNA When placed in culture the sulfate conjugation showed a marked decrease, so that it was down to about 30% after 24 hr, but then started to recover during the next days The glucuronidation activity (MU) also increased after day 2 and remained high for at least two weeks (Fig 2, high activities have been measured up to three weeks, results not shown) MP had no significant effect on the conjugation reactions With pNP as substrate the activity in homogenates of freshly isolated cells was 346 7 pmol/min/μg DNA, and it remained at this

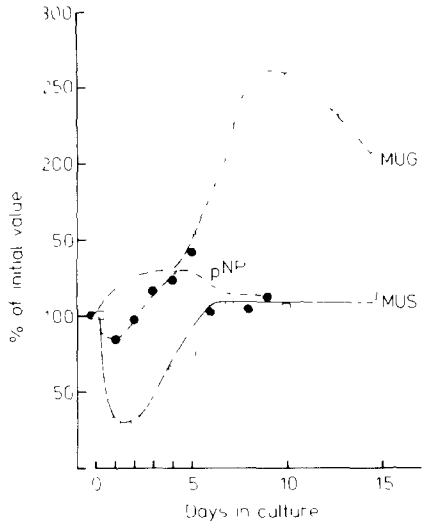

Fig 2 Glucuronidation (MUG, △---△) and sulfation (MUS, □——□) of MU in whole cells and GT (pNP ●— — —●) in cell homogenates as a function of culture time The activities in freshly isolated cells are taken as 100%, and were (±S E) 23 5 (±3 7, N = 9) pmol/min/μg DNA for MUG, 7 2 (±1 5, N = 9) pmol/min/μg DNA for MUS and 346 7 (±15, N = 4) pmol/min/μg DNA for GT(pNP) The values are means of 5–10 separate experiments (2 determinations per point in each), the S E between different experiments ranged from 4 to 25% For details see Materials and Methods

level for at least a week (pNP conjugating activity was somewhat variable in older cultures), with a transient decrease on day 1, followed by recovery to initial values (Fig 2)

The ECDE activity in homogenates of freshly isolated cells was 16 0 pmol/min/μg DNA In cells cultured in control medium the activity declined rapidly, with a significant loss already 4 hr after plating, and by 24 hr more than 50% of the activity was lost (see Fig 3) Addition of MP effectively slowed

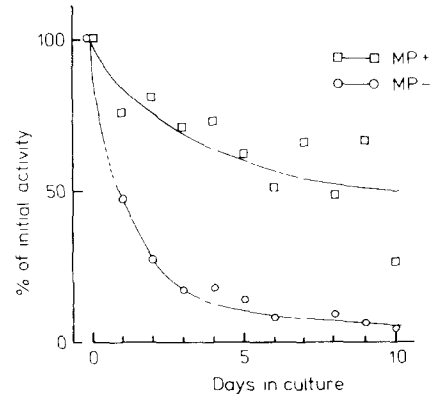

Fig 3 ECDE activity as a function of culture time Cells were grown either in the absence (MP−) or presence (MP+) of 0 5 mmol/l MP, harvested at indicated times and assayed for ECDE as described in Materials and Methods The values are means of 3–6 separate experiments (2 determinations per point in each) The activity (±S E) in freshly isolated cells was 16 0 (±2 5, N = 7) pmol/min/μg DNA The S E between different experiments ranged from 2 to 14%

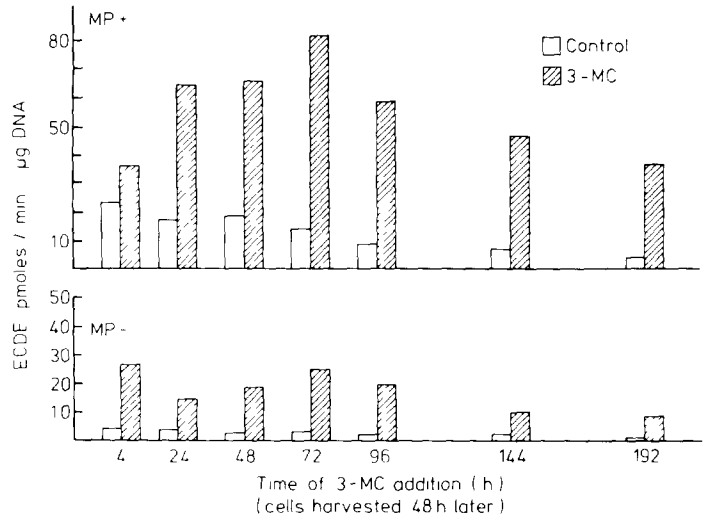

Fig 4 Induction of ECDE by MC as a function of culture time Cells were grown either in the absence (MP−) or presence (MP+) of MP and MC was added at indicated times The cells were harvested 48 hr later and assayed for ECDE as described in Materials and Methods Results from one representative experiment are shown

the decline, so that a 50% loss was seen only after 6 days. After 10 days, cells grown in the presence of MP had 26% of the activity left, whereas it was only 8% in cells grown without it.

Effect of inducers. MC induction of ECDE activity in cultures of different ages is shown in Fig 4 The inducibility varies with culture time, but does not correlate with the basal activity There is little induction in the beginning, and the cells are most responsive to inducer on days 2–3, with highest induced activity on days 4–5 (this is seen if the inducer is present the whole time or if it is added 48 hr before harvesting the cells) After 5 days the activity declines even if inducer is present The presence of MP does not affect the time course of the induction, even though the activities in its presence are higher both in control and MC cultures Maximum induction is 10-fold in cultures without MP and 7-fold in its presence.

The induction of ECDE by PB was similarly dependent on the age of the culture Little induction was seen during the first days and maximum induced activity was seen after 4–5 days; it was 8-fold in the absence of MP and 4-fold in its presence (Fig 5)

The induction of GT (pNP) by MC showed a similar dependence on the age of the culture, although the decline in older cultures was less pronounced. In the beginning of the culture little response to inducer was seen, and maximum induced activity, 2.5 times the basal one, was measured on day 6 The activity on day 9 in the presence of inducer was still twice the basal one (Fig. 6)

DISCUSSION

In the design of the present experiment optimal conditions for the hepatocyte cultures were sought by using media and supplements that have been

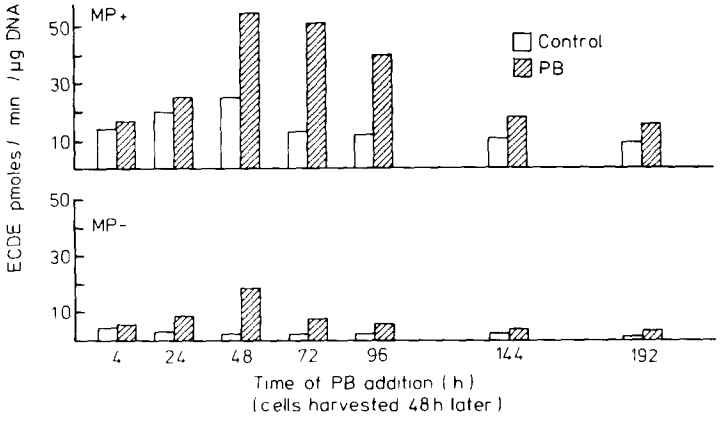

Fig 5 Induction of ECDE by PB as a function of culture time Cells were grown either in the absence (MP−) or presence (MP+) of MP and PB added at indicated times The cells were harvested 48 hr later and assayed for ECDE as described in Materials aned Methods Results of one representative experiment are shown

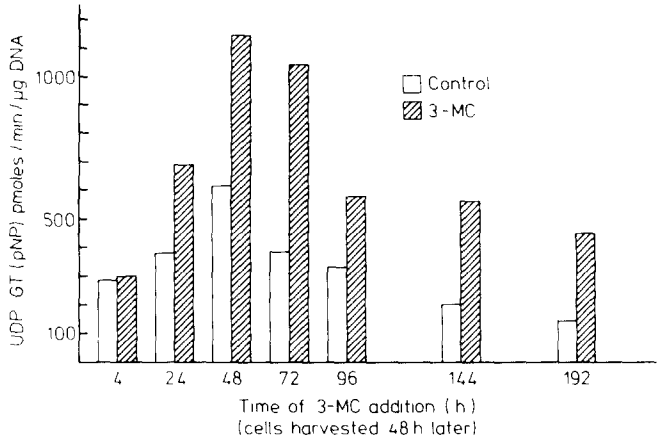

Fig. 6 Induction of GT (pNP as substrate) as a function of culture time MC was added at indicated times, the cells harvested 48 hr later and assayed for GT as described in Materials and Methods Results from one representative experiment are shown

reported to improve survival and preservation of hepatocytes in culture [6, 11–15] The hepatocytes were grown on collagen gels and the medium was a mixture of Ham F-12 and Waymouth MB 752/1, which complement each other with respect to many nutritional factors, e.g vitamins and trace elements, supplemented with serum, insulin, glucagon and dexamethasone The cells could be grown for up to three weeks without any significant loss of DNA after the initial loss that probably is due to non-viable cells, which are loosely adhered to other cells and are washed out with medium changes There are relatively few reports in the literature on cultures that have been carried on for more than a few days, during which time the loss of DNA is not truly a reflection of cell death Michalopoulus and Pitot [6] and Tanaka *et al* [14] report DNA/cell time curves similar to our findings for 8-day cultures

ECDE activity was rapidly lost during culture, as has been well documented for cytochrome P-450 in many studies The loss of activity in the absence of MP is about the same as that found by Edwards *et al* [11] in a modified Waymouth MB 752/1 medium Decad *et al* [1] reported maintenance of cytochrome P-450 in hormone supplemented medium, but they did not carry the cultures beyond 24 hr The modifications of Paine *et al* [2, 3] prevent the loss of cytochrome P-450 during 24 hr, but not later unless MP is added, in which case 1 week cultures still have 75% of the activity A similar omission of cystein and cystin and addition of delta-aminolevulinic acid did not prevent a loss of the activities measured by Holme *et al* [16] It is thus obvious that the factors, which would help to maintain cytochrome P-450, are still unidentified

The present study demonstrates that the behaviour of the conjugating enzymes differs quite markedly from that of the monooxygenases There is a large initial decline in sulfate conjugation, but the activity starts to recover after the second day Glucuronidation may show a slight decrease, but then there seems to be an increase in the activity, and GT (MU) is above control values for at least 2 weeks, and GT (pNP) for at least 1 week The large decrease seen in sulfate conjugation could be due to a loss of either sulfotransferase itself, which is a cytoplasmic enzyme in contrast to GT, or it could be loss of factors needed for sulfate activation On the other hand, changes in GT are also taking place, maybe reflecting membrane changes, and a more detailed investigation should be done, e g by measuring detergent activation Even though the glucuronide/sulfate ratio during culture is relatively stable [17], it has to be interpreted with care because of low sulfation in the beginning of the culture and elevated glucuronidation in older cultures

Few studies have been carried out on conjugating enzymes in cultured hepatocytes. Holme *et al* [16] used several media formulations and measured both monooxygenases and GT (pNP) after 22 hr and found a decline in the latter of almost 50%, but they did not measure it in older cultures A loss of conjugating activity during the first hours after isolation has also been reported by Schwarz *et al* [18] Emery *et al.* [19] have reported an increase in glucuronidation during the first 48 hr in culture, although not in sulfation and glutathione conjugation

Poor induction of cytochrome P-450 by PB in the beginning of hepatocyte cultures has also been reported by Newman and Guzelian [20], and Edwards *et al* [11] showed an increase in the induction of ECDE by both PB and MC during the first days of culture The present study demonstrates that also GT(pNP), which is considered to be induced in coordination with the MC-inducible cytochrome P-450 [21, 22], can be induced in culture, and the same effect of culture age is seen, i.e the impaired induction in the beginning of the culture is not specific for cytochrome P-450 and apparently unrelated to the loss of it The lack of induction in the beginning of the culture is possibly a reflection of "stress" caused by the isolation process, when hepatocytes are subjected to enzymatic digestion and detached from their contact with other cells, as well as by the adaptation to culture conditions Membrane changes in freshly isolated cells have been demonstrated [23], and hepatocytes are in a protein

catabolic state after isolation [24]. After about 24 hr in culture the rate of protein synthesis starts to increase ([14]; own unpublished results), and several other hepatic functions show similar recoveries, e.g. insulin stimulation of glycogen synthesis is seen only after 2 days in culture [15] The impaired induction seen in the beginning of the cultures may be a reflection of the impaired protein synthesis, as induction involves synthesis of new enzyme protein. If the inducers are added at the optimum time inductions of both cytochrome P-450 and GT of the same magnitude as *in vivo* can be seen. Ethoxycoumarin, which was used as substrate for the monooxygenase activities, is metabolized by several isozymes of cytochrome P-450, and measuring overall activity does not give information about possible qualitative changes

The effect of isolation and placement in culture on hepatocytes is thus complex. Some of the changes may be irreversible, as at least now seems to be the case with cytochrome P-450, and others are reversible, but no systematic study has been carried out. For studying glucuronidation and enzyme induction primary cultures seem to be optimal during 2–5 days, whereas sulfation is low during this period. The results of the present study emphasize the importance of monitoring the culture over several days in order to establish an optimum time for each specific parameter This ensures that the results are more physiological and consistent

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