Hormone- and endotoxin-modulated gene expression of a long-term organ culture system of adult rat liver

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Abstract Precision-cut slices of normal adult rat liver maintained in serum-free medium remain hormone- and endotoxin-responsive for at least 48 h. They respond to glucocorticoid (dexamethasone) with the induction of the gluconeogenic enzyme tyrosine aminotransferase (TAT), as determined by enzymatic activity and by the increase in enzyme protein. Furthermore, endotoxin (LPS) induced nitric oxide synthase II (i-NOS), and this induction is repressed, similarly to the in vivo situation, by dexamethasone (DEX). All increases are inhibited by cycloheximide (CHX). The length of the period of responsiveness suggests that this organ culture system might be generally useful for studying the modulation of liver gene expression by physiological and pathological influences.

Key words: Precision-cut liver slice; Long-term organ culture; Tyrosine aminotransferase induction; Endotoxin; Nitric oxide synthase repression; Gene expression

1. Introduction

The complexity of liver structure and the multiplicity of metabolic, homeostatic, endocrine and detoxification functions performed by the organ [1] have resulted in numerous efforts to develop in vitro systems that allow the study of single functions under controlled environmental conditions. These systems all share the characteristic that they eliminate the influence of other organs and of the circulatory system, thus providing conditions intermediate between 'test tube biochemistry' and the in vivo situation. However, most of these systems also share the characterstic of being immersed in the highly variable [2] medium component fetal calf serum.

Improvements in the standardization of primary cultures of hepatocytes have allowed the analysis of a number of reactions performed by these cells, and of their response to physiological and pathological (e.g. xenobiotic) stimuli [3]. These developments have also allowed to study specific functions of the other several cell types of the normal liver and aspects of their interactions with hepatocytes [4].

Functionally differentiated hepatocyte cultures have a limited life span and, in spite of improvements in the maintenance of differentiated properties by additives to the culture medium, components of the extracellular matrix or by different forms of coculture, a decline of liver specific functions cannot be prevented [3,5,6]. Furthermore, the advantages of pure-cell systems are endowed with the problem of missing organotypic cell–cell and cell–ECM interactions [7]. Therefore, results with these systems cannot be directly extrapolated to the in vivo situation.

To overcome these problems, in vitro systems have been developed, in which the organ structure is at least partially maintained for a certain time [8]. In practice, however, difficulties in ensuring an adequate nutrient and oxygen supply leads to short survival times and a severe loss of specific liver functions. Among these more complex, organotypic systems, thin liver fragments, 'slices' [9], are endowed with some advantages: metabolites and substances to be tested are eliminated and can reach the cells by diffusion, and a relatively large number of tests might be performed with material from a single liver. Thus, the development of precision-cut liver slices [10] appears to have overcome some of the major limitations of other organotypic systems, even though resorting to the efficient, but artificial nutrient and oxygen supply by diffusion, as opposed to the circulatory system [3].

In spite of the limitations, the obvious advantages of the system and a series of interesting experimental set ups [11,12] have made it very attractive. As a consequence a large number of publications have been devoted to the description of its viability on the basis of measurements of general cellular parameters (e.g. intracellular K^+ and ATP, incorporation of [^{3}H]leucine, MTT-reduction, LDH release [13]).

By comparison, the study of liver-specific functions has proven to be less forwarding, and the problem of short useful periods (8 h [14]) has not yet been satisfactory solved. In some cases a pretreatment of the animals has yielded 'liver-specific' in vitro results (e.g. with LPS [15], aroclor 1254 [16], phenobarbital [17] or adrenalectomy [18]). These approaches do not provide an alternative for studies requiring large numbers of laboratory animals. In an attempt to overcome some of these limitations, we have established an organ culture system, in which liver-specific hormone responsiveness and the capacity to react in a liver-specific manner to bacterial endotoxin (LPS) was maintained for at least 48 h.

2. Materials and methods

2.1. Animals

Male Lewis rats (Charles River), with a body weight of about 275–300 g were housed under controlled environmental conditions with a defined light and darkness cycle, and with unrestricted access to food and water. To avoid variability resulting from circadian rhythms of liver functions the preparations were always performed at the same time of the day. All experiments were performed in agreement with the German guidelines for the care of laboratory animals.

2.2. Organ culture

The animals were killed by CO_2 lethal narcosis. After exsanguination the livers were immediately removed and kept in ice-cold (4°C) PBS (phosphate-buffered saline) until slicing. After rinsing thoroughly in several changes of buffer, liver lobes were trimmed into blocks of 1×1.5 cm, from which slices of 250–300 μm thickness were cut using an automatic tissue slicer (Science Services, Munich). Slices were collected in cold (4°C) L-15 medium supplemented with 10% fetal calf

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serum followed by a 24 h pre-incubation in L-15 supplemented with 10% FCS, 4 mM L-glutamine and 15 mM Hepes buffer (pH 7.2). All incubations were performed in media without antibiotics. After the 1-day pre-incubation, experiments were continued in serum-free L-15 medium and, where indicated, the medium was supplemented with dexamethasone (DEX; 25–50 μM), LPS (20 μg/ml; 0111:B4 Sigma, Deisenhofen, Germany). Unless indicated otherwise all chemicals were purchased from Sigma.

2.3. Enzyme activity

Determinations of TAT (EC 2.6.1.5.) activity were performed as described [19]. Specific activity was expressed as the amount of enzyme forming 1 μmol of *p*-hydroxyphenylpyruvate/min/mg protein at 37°C. Protein concentration was measured by the Bradford method with BSA as a standard [20].

2.4. Western blotting

For immunoblotting the slices were homogenized in eight volumes of reducing sample buffer and boiled for 10 min at 90°C. After separation on 5–15% PAA gels the proteins were blotted onto nitrocellulose (Schleicher and Schuell) and detected with ECL (Amersham) as recomended by the supplier. Equal amounts of proteins per slot were confirmed by staining the blots with Ponceau-S [21]. Tyrosine aminotransferase (TAT) was detected with a polyclonal rabbit antibody against the rat enzyme (a kind gift of G. Schütz, DKFZ, Heidelberg) and NOS-II was demonstrated with a monoclonal antibody against i-NOS from macrophages (Transduction Laboratories, Lexington, KY). Analysis of the scanned blots was performed with a recently developed MacIntosh program [22].

3. Results

3.1. TAT activity and inducibility

As shown in Fig. 1 the serum-free organ culture system responds readily to glucocorticoids with a time-dependent increase in TAT activity. The increase in enzymatic activity is prevented by cycloheximide (CHX), indicating its dependence on new protein synthesis. The induction proceeds more slowly than in vivo (Fig. 2), but the capacity of the system to respond to the hormone is similar to the organ in vivo, as shown by the activity obtained after longer incubation times and after a brief 'protective' [23] pretreatment with the glucocorticoid. The induction was further confirmed by the increase in the amount of the 54 kDa TAT protein, specifically detected with the polyclonal antibody against the purified enzyme, and the prevention of this increase by cycloheximide (Fig. 3).

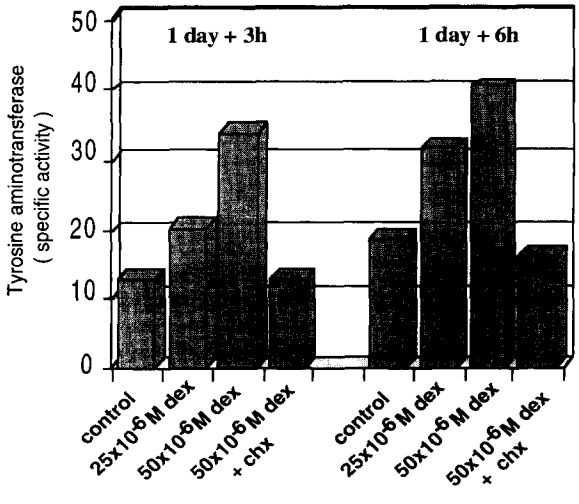

Fig. 1. Glucocorticoid induction of TAT in liver slices during the second day of culture. Enzyme activity was measured 3 and 6 h after adding DEX (25 and 50 μM). CHX (8 μg/ml) was added to the incubation with the higher hormone concentration. All treatments were started after the 24 h pre-incubation. Results are from four independent experiments.

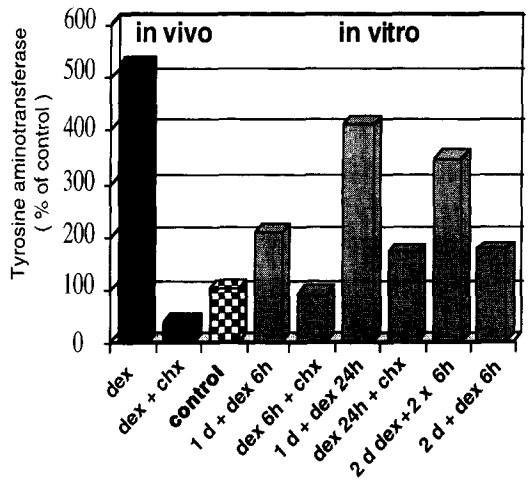

Fig. 2. Comparison of TAT expression in vivo and in long-term organ culture. The in vivo induction was measured 6 h after an i.p. injection of DEX (3 mg/kg) and i.p. DEX + CHX (50 mg/kg). The in vitro results correspond to slices treated for 6 and 24 h with DEX (50 μM) and, in addition, to slices pre-incubated for 6 h in the presence of the hormone followed by a 18 h incubation in serum-free medium and then again for 6 h with DEX (50 μM). All treatments were started after the 24 h pre-incubation. Data are expressed as percent of control. Mean specific activities in vivo: 21.9 and in vitro (24 h incubation): 10.3 μmol/min/mg protein, respectively.

The liver is also a major target organ for endotoxin [24], but the data obtained with primary cultures of hepatocytes do not adequately represent the complex interactions that occur in vivo [25]. The long-term liver organ culture system shows the typical liver gene expression response to endotoxin with induction of nitric oxide synthase II (NOS-II) and also the prevention of this induction by dexamethasone (Fig. 4).

These results indicate that in this serum-free organ culture system both induction and repression of gene expression can proceed with results comparable to the in vivo situation.

4. Discussion

The results now reported show that precision-cut slices of normal, untreated adult rat liver can be used for the study of liver-specific functions for at least 48 h, as demonstrated by the induction of TAT and the induction and repression of NOS. The conditions achieved provide stable organ cultures well beyond the reccommended [14] first 8 h after obtention of the slices, a period during which some liver functions may be altered by the tissue processing conditions.

TAT was chosen as one of the most extensively studied examples of liver-specific hormone responsive systems [26]. Expression of the enzyme begins in the rat shortly after birth. In culture its expression can be maintained for some time by providing the primary hepatocytes with a number of hormones [27] and with extracellular matrix [28,29]. In contrast to other hormonal studies [30] the changes in TAT activity depend on a de-novo synthesis of the enzyme, thus providing an excellent parameter for determining intact protein synthesis, 'hepatic quality' and hormone-modulated gene expression at the same time [31,32]. The somehow slower than in vivo reaction to the modulators is assumed to be a consequence of

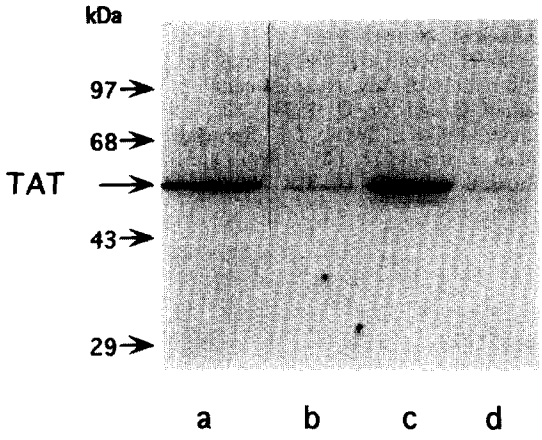

Fig. 3. Immunoblot demonstration of TAT induction. (a) Homogenate from adult rat liver. (b) Untreated liver slices incubated for 24 h (control). (c) Slices treated with DEX (50 μM) for 24 h. (d) Slices treated as for (c) but supplemented additionally with CHX (8 μg/ml). All treatments were started after the 24 h pre-incubation. TAT immunoblots were performed routinely for all experiments.

the in vitro situation with exchange of metabolites and substrates by diffusion, as opposed to the in vivo situation, with an intact circulatory system. In its present state of development the system provides conditions for gene expression studies without the need of large numbers of experimental animals.

The inducible form of NOS (i-NOS/NOS-II) can represent the predominant enzyme isoform in the liver [33]. Its pathophysiologic importance in endotoxemic, antimicrobic and antitumorigenic events is rapidly becoming apparent [34]. Current evidence indicates that in addition to the cells of the reticuloendothelial system and hepatocytes, presumably also some of the other cell types of the organ can either express or modulate the expression of the enzyme. However, the general picture is still incompletely understood [35]. The organ culture system might, therefore, contribute to a better understanding of some functions of hepatic NOS under controlled conditions, while maintaining organ specific cell–cell and cell–ECM interactions and without resorting to large series of animal experiments. In the serum-free culture system endotoxin (LPS) induces i-NOS directly and not only at standard (10–20 μg/ml) but also at much lower (2 μg/ml) concentrations (unpublished results). In contrast, standard cell culture systems have been reported to require additional treatments with cytokines [36], macrophage-conditioned medium, Kupffer cell co-cultivation or hepatocytes prepared from endotoxin-pretreated animals ('primed hepatocytes' [33,36]). These requirements are obviated with our organ culture system which, as now described, can provide from a single liver a large number of samples for the study of induction (TAT, i-NOS) and repression (i-NOS) of gene expression.

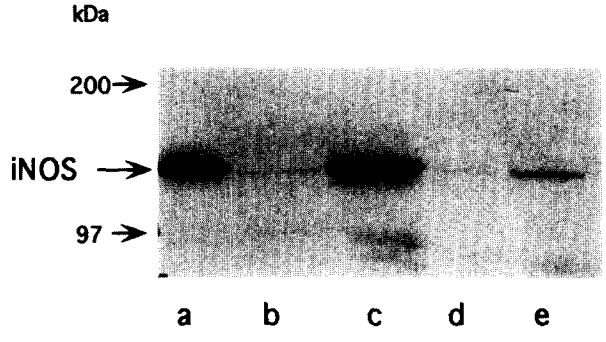

Fig. 4. Expression of i-NOS in long-term slice culture of normal adult rat liver. (a) Positive control (130 kDa i-NOS) from induced macrophages (Transduction Laboratories). (b) Liver slices cultured for 24 h without additions (control). (c) Slices incubated for 24 h with LPS (20 μg/ml). (d) Slices incubated for 24 h with LPS + CHX (8 μg/ml). (e) Slices incubated for 24 h with LPS + DEX (50 μM). All treatments were started after the 24 h pre-incubation. The results shown are representative of four independent experiments.

In summary, in its present state of development, the long-term organ culture system allows the analysis of gene expression phenomena modulated by glucocorticoids and endotoxin. The near in vivo efficiency at which the reactions occur suggests a wider applicability of this organ culture system for the study of liver reactions to physiological and pathological influences.

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