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Application of the Hepatocytes Bioreactor to Xenobiotic Biotransformation

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Summary

This study deals with the application of the previously developed immobilized and perfused isolated hepatocytes as a cellular system for the study of representative phase I and phase II of biotransformation reactions. To illustrate phase I reactions, aminopyrine (0.17–4.25 mmol/l) and hexobarbital (0.2 mmol/l) were selected. For phase II reactions, glutathione transferase activity was evaluated by using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate (0.125–2.0 mmol/l). Formaldehyde, that was formed from aminopyrine, increased steadily in the perfusion medium with time. The perfused hepatocytes eliminated hexobarbital at a much higher rate than the hepatocytes in suspension. At several time points the amount of CDNB-glutathione conjugate formed per one million hepatocytes in the bioreactor was almost twice the amount formed by the hepatocytes in suspension. The present data illustrate the successful application of the hepatocyte bioreactor in phase I and phase II of xenobiotic metabolism and indicate that the cells were metabolically more active than the cells in suspension.

Key words

Aminopyrine-1-chloro-2,4-dinitrobenzene – Hexobarbital – Hepatocytes – Xenobiotic biotransformation

Introduction

The liver is the most important and unique organ in all metabolic processes including both eubiotics and xenobiotics. This is due to the fact that the liver is possessing very high nonspecific enzymatic activities towards individual substrates. These enzymatic activities, however, may be considered specific towards certain chemical groups. All metabolic enzymes are genetically determined in the organism and are affected by both exogenous and endogenous factors, e.g. age, sex, nutritional status, species, environmental factors and the pathological state of the organism (Daniel 1982, Sitar 1989, Barry and Feely 1990, Yang *et al.* 1992). Depending on the specific chemical structure of the foreign compound, xenobiotic biotransformation, which predominantly takes place in the liver cells, can be described to occur in two phases. In phase I, the major reaction involved is hydroxylation catalyzed by members of the class of enzymes, known as monooxygenases or cytochrome P-450 species. In addition, phase I includes reduction and hydrolytic reactions which modify the pharmacological activity of the drug. In phase II, the hydroxylated or other products of phase I are converted, by specific enzymes, to various polar metabolites by conjugation. The purpose of the two phases of xenobiotic metabolism is to increase their polarity and facilitate their excretion from the body. The monooxygenase system contains many forms which vary in their activity towards various xenobiotics (Murray 1991).

Goal and experimental protocol

It is well established that isolated liver cells contain all the necessary cofactors for all types of metabolic activities. There is no need to add the cofactors as in the case when working with subcellular organelles, e.g. microsomes and mitochondria. After

isolation of hepatocytes by the standard collagenase method (Moldeus *et al.* 1978), this investigation, therefore, is directed toward the application of the developed immobilized and perfused isolated hepatocytes as a cellular system for the study of representative phase I and phase II biotransformation reactions. Simultaneously, analogous biotransformation study was conducted on hepatocytes in suspension to compare the two cellular systems. To illustrate the phase I reactions, two substrates, namely, aminopyrine (amidopyrine) and hexobarbital were selected. Furthermore, glutathione transferase activity was evaluated by using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. During phase I, aminopyrine is N-demethylated by aminopyrine N-demethylase leading to the formation of either the monomethylated product or the completely demethylated product in two successive steps. Formaldehyde is the direct product of demethylation. Therefore, two molecules of formaldehyde are formed from one molecule of aminopyrine. Formaldehyde was evaluated colorimetrically by the Nash reagent (Kupfer and Levin 1972). Moreover, during phase I, hexobarbital hydroxylation was assessed. Hexobarbital hydroxylation is catalyzed by alicyclic hydroxylase leading to the formation of 3-hydroxyhexobarbital which in turn is changed to 3-ketohexobarbital by the cytosolic dehydrogenase. The rate of hexobarbital oxidation was estimated from the time of the disappearance of unchanged hexobarbital in the relevant medium. The medium contained a known initial concentration of the parent compound and the gradual changes in the latter were estimated spectrophotometrically. For glutathione conjugation the cummulative time course of the synthesis of CDNB glutathione conjugate was followed up in the medium. The resulting CDNB conjugate by the liver cells was measured in the medium by ultraviolet spectrophotometry according to Habig and Jacoby (1981). Several initial concentrations were used ranging from 0.125 mmol/l up to 2.0 mmol/l.

Results and Discussion

The time course of formaldehyde formation was observed after two initial aminopyrine concentrations. The lower one was 0.17 mmol/l while the higher concentration was 4.25 mmol/l. Fig. 1 demonstrates that, with regard to the lower concentration, the formed formaldehyde exhibited practically a steady concentration due to the continuous removal of the formed metabolite to the next stage (in which formadehyde is oxidized to formic acid and the latter to CO_2). Formaldehyde, that was formed at the higher concentration of aminopyrine, increased steadily in the perfusion medium with time. This may mean that the next oxidation step was not sufficient to handle all the formed formaldehyde and resulted from the initial concentration of 4.25 mmol/l aminopyrine. The disappearance of hexobarbital in the perfusion medium of the immobilized hepatocytes as compared to the well shaken and oxygenated hepatocyte suspension as a function of time is demonstrated in Fig. 2.

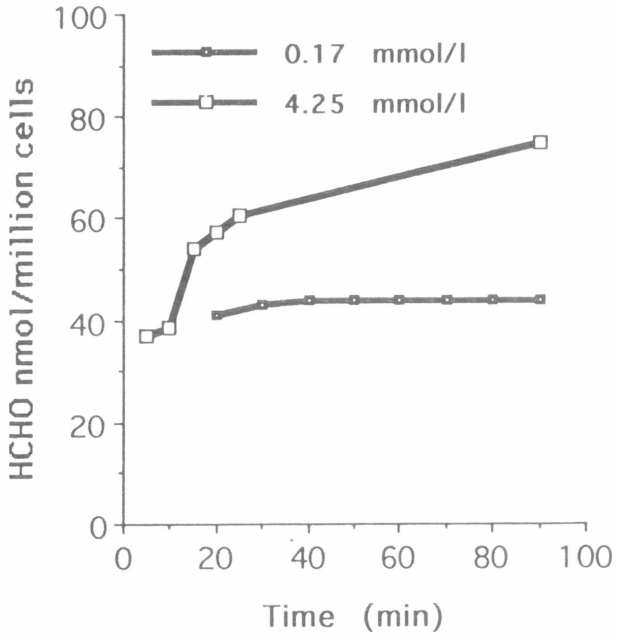

Fig. 1
The time course of formaldehyde formation in the medium of perfused immobilized hepatocytes.

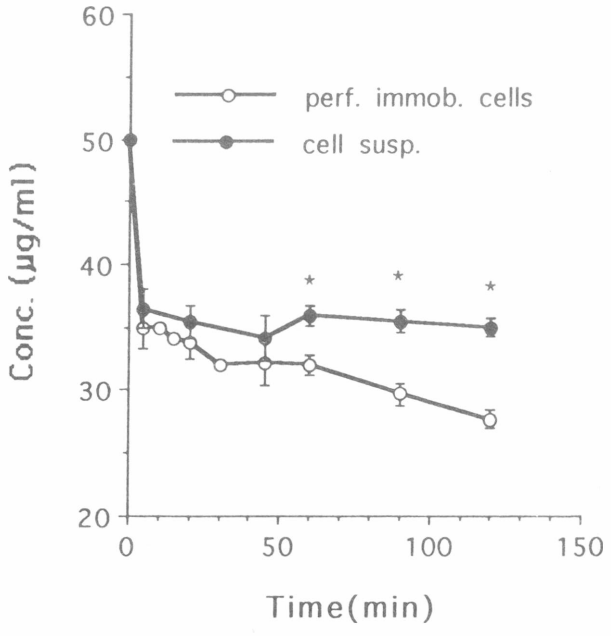

Fig. 2
The time course of hexobarbital disappearance in the media of perfused immobilized hepatocytes and of the hepatocyte suspension (mean ± S.E.M., n=5). * indicates significantly different values ($p<0.05$).

The kinetics of hexobarbital disappearance, as depicted from the last figure, were obtained under exactly the same conditions and simultaneously. It is evident that the immobilized and perfused hepatocytes eliminated hexobarbital at a much higher rate than hepatocytes in the suspension. The hexobarbital concentration was significantly lower in the perfusion medium than in the cellular suspension medium at various time intervals.

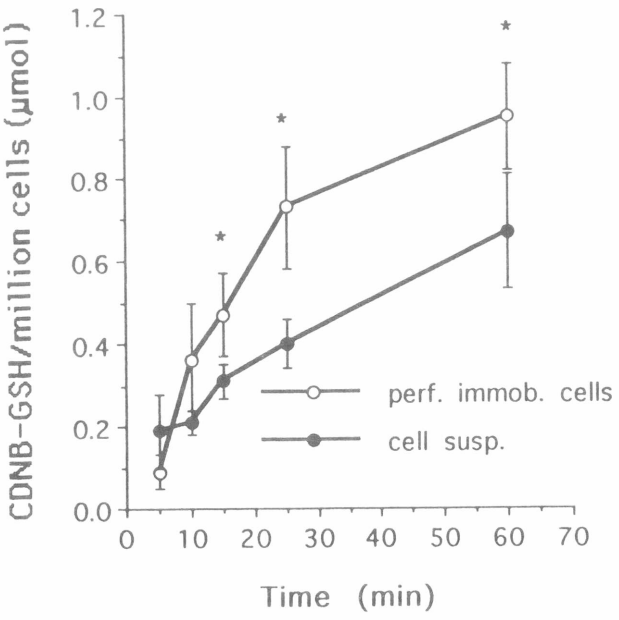

Fig. 3
The time course of the cummulative formation of CDNB-GSH conjugate by the immobilized perfused hepatocytes and hepatocytes in suspension (mean ± S.E.M, n=5). * indicates significantly different values ($p<0.05$).

Glutathione transferase activity was studied by using CDNB conjugation activity after four initial CDNB concentrations (0.125, 0.5, 1.0 and 2.0 mmol/l) in the perfusate of the immobilized hepatocytes. The amount of CDNB-glutathione (CDNB-GSH) conjugate per million cells in the bioreactor was calculated. The amount of CDNB-GSH increased in time and was concentration-dependent. If we plot the rate of CDNB-GSH formation versus the initial concentration of CDNB 10, 20 or 25 min after CDNB addition, Michaelis-Menten kinetics could be predicted. To compare the metabolic efficiency of the immobilized cells in the bioreactor with the hepatocytes in suspension, we have used the intial concentration of 1.0 mM for CDNB. The rate of formation of CDNB-GSH conjugate per million cells was followed up for 60 min (Fig. 3). For hepatocytes both in suspension and in the form of the bioreactor there was a continuous increase in the amount of the conjugate with progressing time. There was, however, a significant difference related to the time course of both curves. At several time points, the amount of CDNB-GSH formed per one million hepatocytes in the bioreactor was almost twofold higher than the amount formed by the hepatocytes in suspension.

The conclusion here is straightforward regarding the successful application of the bioreactor in phase I and phase II of xenobiotic metabolism. Another advantage in the perfused immobilized cell model is the potential possibility to use the bioreactor repeatedly for several experiments at relatively short periods of time assuming that sufficient time has elapsed between each application to allow for cellular stabilization. In addition, the results of each experiment are much less variable and better reproducible than in the cellular suspension due to the inherited simplicity and good handling of the cells with less possibility of technical error or mechanical damage. Due to the more physiological condition of the hepatocytes in the bioreactor, xenobiotic biotransformation was faster for both phases I and II representative susbstrates in the present study. The results of a better xenobiotic biotransformation by cells in the bioreactor, reported herein, are in harmony with our previous results of the perfused and immobilized hepatocytes which are at a high energy state and are more resistant to various kinds of chemical insults.

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Reprint Requests

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