Differential sensitivity of rat hepatocyte CYP isoforms to self-generated nitric oxide

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Abstract Early loss of P450 in rat hepatocyte cultures appears directly related to nitric oxide (NO) overproduction. This study investigates the influence of endogenously generated NO (or NO-derived species) on the relative expression of cytochrome P450 (CYP) isoforms in rat hepatocytes. Our results support the view that loss of P450 holoenzyme in culture is the ultimate consequence of a NO driven process, activated during the common hepatocyte isolation procedure, that leads to an accelerated and selective degradation of specific CYP apoproteins. Under conditions in which NO and peroxynitrite formation is operative, changes in the level of specific CYP isoforms result in a significant alteration of the CYP apoprotein profile that after 24 h of culture is quite different from that found in the liver of uninduced rats. This process is reverted by the early and efficient inhibition of NO synthesis, which allows for (1) maintenance of total P450 holoenzyme content, (2) preservation of the initial constitutive CYP pattern in culture and (3) the early expression of the normal inducibility in response to model inducers.

Key words: Nitric oxide; Drug metabolism; P450 content; Cytochrome P450 isoform; Cytochrome P450 induction; Rat hepatocyte culture

1. Introduction

Loss of cytochrome P450 (CYP)-dependent metabolism during the early hours of hepatocyte culture is considered the most serious limitation of this model for in vitro studies of drug metabolism, drug–drug interactions, long-term toxicity and P450 induction [1,2]. In a mechanism-based approach, we have recently demonstrated [3] the direct involvement of self-generated nitric oxide (NO) as being responsible for the phenotypic instability of rat hepatocytes in short-term culture. Activation of constitutive nitric oxide synthase (cNOS) and oxidative stress during the cell isolation stages leads to formation of significant amounts of peroxynitrite (PN) in the intracellular environment from the simultaneous flux of NO and superoxide [4]. Our conclusion was that both rapid loss of P450 and transient disturbances on aerobic mitochondrial function in short-term culture should ultimately be a consequence of a NO-driven common mechanism involving the early interaction of NO (or NO-derived species, likely PN) with the hepatocyte haemoprotein pool [3–5]. In conventional monolayer cultures, the early loss of P450 can fully be avoided by an efficient inhibition of cNOS that allows the preservation of catalytically active P450 to levels close to those of the intact liver [3].

The mechanism(s) underlying NO interaction with pre-existing P450s [6–10] suggest that NO is a non-specific inactivator of these haemoproteins. However, owing to intrinsic differences in haem accessibility, protein structure and/or turnover, NO may well differentially affect inducible or constitutive CYPs. This work studies the influence of endogenously generated NO on the relative expression of P450 isoforms in hepatocytes, by analysing the evolution of the CYP apoprotein profile in standard and N^G-nitro-L-arginine methyl ester (L-NAME)-treated cultures. Results show that the inhibition of NO synthesis not only preserves the initial P450 holoenzyme content but also ensures the basal expression of the constitutive CYP pattern initially present in the intact liver.

2. Materials and methods

2.1. Cell isolation and culture

Experiments were performed in conventional monolayer culture, expressly avoiding the use of complex culture substrata or medium supplements known to contribute to a better preservation of P450. Hepatocytes were isolated from fed male Sprague–Dawley rats (180–250 g) by two-step collagenase liver perfusion and cultured on fibronectin-coated dishes, as described [5]. In standard cultures, cells were maintained in Ham's F12 medium supplemented with 0.2% bovine serum albumin, 10^{-8} M insulin and dexamethasone (DEX), 2% newborn calf serum (Gibco) and antibiotics. In parallel experiments, hepatocytes were isolated and cultured by the same procedure except for the addition of 1 mM L-NAME (Sigma) to the perfusion and culture media (L-NAME-treated cultures). Unattached cells were removed by renewing the medium 1 h after plating. Viability (trypan blue dye exclusion test) always exceeded 95%.

2.2. In vitro induction protocol in cultured rat hepatocytes

DEX and β-naphthoflavone (BNF) were added to the culture medium to a final concentration of 50 μM. To avoid disturbances on cell attachment, L-NAME-treated cultures were allowed a period of 6 h after plating before the beginning of exposure to inducers. At 24 h monolayers were shifted to fresh medium (now free from serum and L-NAME) containing the inducers, and exposure was prolonged for up to 48 h.

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Abbreviations: NO, nitric oxide; cNOS, constitutive nitric oxide synthase; PN, peroxynitrite; L-NAME, N^G-nitro-L-arginine methyl ester; CYP, cytochrome P450; DEX, dexamethasone; BNF, β-naphthoflavone

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2.3. Cell subfractionation, electrophoresis and Western blot analysis of P450 isoforms

Isolated hepatocytes and monolayers were frozen under liquid N_2 and kept at −20°C until used. Microsomes were prepared by centrifugation (100 000 × *g*, 90 min) of the S9 fractions from crude homogenates (5–10 mg protein/ml) obtained by brief sonication on ice-cold 0.1 M potassium phosphate/1 mM EDTA, pH 7.4. The pellets were resuspended in 50 mM Tris–HCl/20% glycerol buffer (pH 7.4), aliquoted and stored at −20°C until used. Proteins (1–20 μg microsomal protein/well) were resolved (SDS–PAGE, 4–15% gradient gels) and transferred to PVDF membranes. Immunodetection was performed with rabbit polyclonal antibodies raised against rat CYP2B1/2 and human CYP1A1, 1A2, 2C9, 2E and 3A4/5 (provided by P.B.) proved able to cross-react with their corresponding rat orthologs. The immunoreactive bands were detected by peroxidase-coupled chemiluminescence (ECL kit, Amersham) followed by densitometric analysis. Quantitative estimation of CYP apoprotein content was based on the interpolation of the signal intensity of microsomal samples in standard curves constructed in the same immunoblot with known amounts of pure CYPs, as described [11]. Data are referred to as mg microsomal protein.

2.4. Other determinations

P450 was determined by the absorption spectra of the reduced form versus the reduced P450–CO complex [12]. Protein was assayed by the Lowry procedure. Experiments were performed in at least three different cultures with 3–4 plates per variable. Data are presented as means ± S.D. Statistical analysis was done by the Student's *t*-test.

3. Results

3.1. Differential effect of endogenous NO on immunodetectable P450 isoforms

We have previously shown [3] that efficient inhibition of NO synthesis is a determinant for the full maintenance of total P450 holoenzyme in short-term cultures. Fig. 1 shows the immunodetection of individual rat liver CYPs (1A1/2, 2B1/2, 2C11, 2E1 and 3A1/2) as a function of isolation and culture conditions. With the exception of CYP1A1, constitutive expression of all the isoforms studied is readily detectable in freshly isolated hepatocytes. After 24 h under standard culture conditions, CYP1A2, 2C11 and 3A1/2 apoproteins significantly decrease to very low levels (less than 20% of the initial value). The 2E1 and 2B1/2 isoforms are also significantly suppressed but to a lesser degree (55 and 36% remaining, respectively). The inhibition of NO synthesis contributes significantly, although to different degrees, to a better preservation of all the CYPs analysed (Fig. 1). Of particular interest is the good maintenance of the 2C apoenzyme (> 75% of the initial content after 24 h) in spite of the fact that this isoform appears to be the most sensitive to NO under standard culture conditions. CYP2B1/2, 2E1, 1A2 and 3A1/2 apoenzymes (Fig. 1) are also well preserved in L-NAME-treated cultures (ca. 90, 80, 60 and 45% of the initial value, respectively).

3.2. CYP apoprotein profile in standard versus L-NAME-treated hepatocyte cultures

We have further analysed the evolution in culture of the initial CYP apoprotein pattern in quantitative terms (Table 1, Fig. 2). Constitutive expression of liver CYPs in Sprague–Dawley rats is known to be highly variable among different animals (see 0-time values). As shown in Table 1, the isolation and culture of hepatocytes under standard conditions lead to a significant decrease in the apoprotein content for all the P450s assayed. However, there appears to be a differential suppression depending on the CYP isoform considered. As Fig. 2 shows, this results in a significant alteration of the apoprotein profile after only 24 h in culture. The very important decrease of the CYP2C apoenzymes together with a better preservation of the 2B isoforms, shifts the initial CYP pattern (typical of uninduced male rat liver) to one more similar to that of hepatocytes from phenobarbital-treated animals. Early exposure to L-NAME efficiently prevents these changes. By 24 h of culture the concentration of the individual

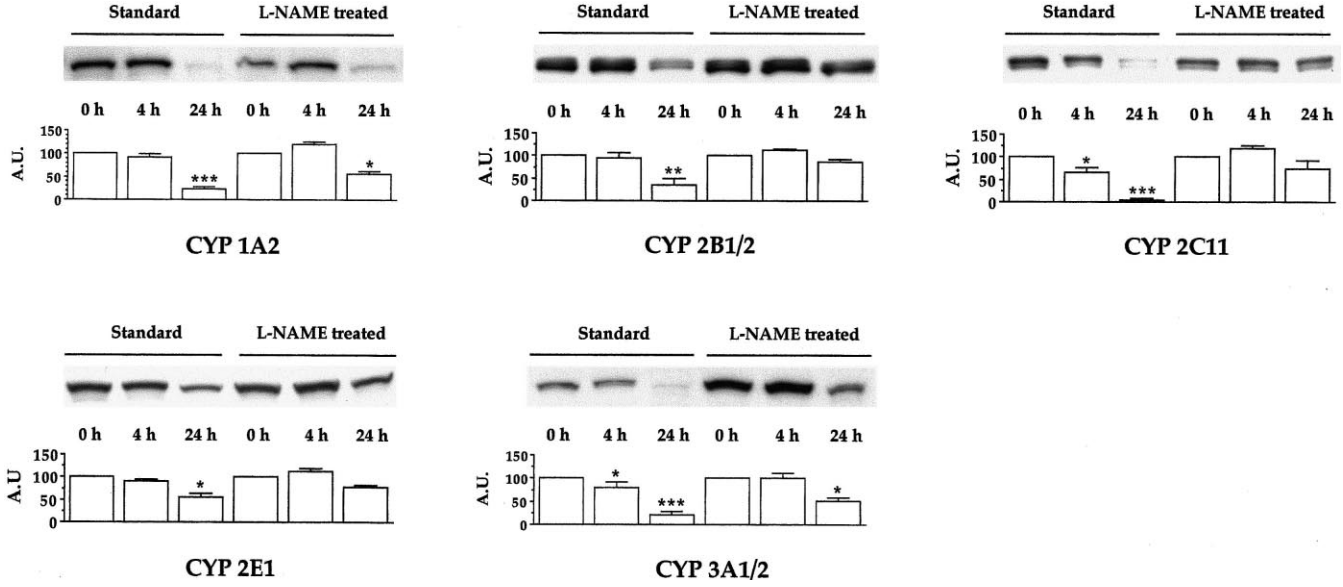

Fig. 1. Western blot analysis of CYP isoforms as a function of time and culture conditions. Equal amounts of microsomal proteins (20 μg/lane for CYP1A, 2E and 3A, 4 μg/lane for 2B and 2 μg/lane for 2C) were processed (one representative experiment is shown). Constitutive expression of 1A1 is almost undetectable and therefore not shown. Two anti-2C9 immunoreactive bands are detected in the 'P450 2C region': the upper (about 51 kDa) corresponds to CYP 2C11, the lower we believe is 2C13. Densitometric analysis (arbitrary units) corresponds to means ± S.D. ($n = 3$ different cultures). Significant decrease relative to their corresponding 0 h value: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. CYP content in 24 h L-NAME-treated cells is significantly increased ($P < 0.05$) as compared to 24 h-old standard cultures for all the isoforms (not shown).

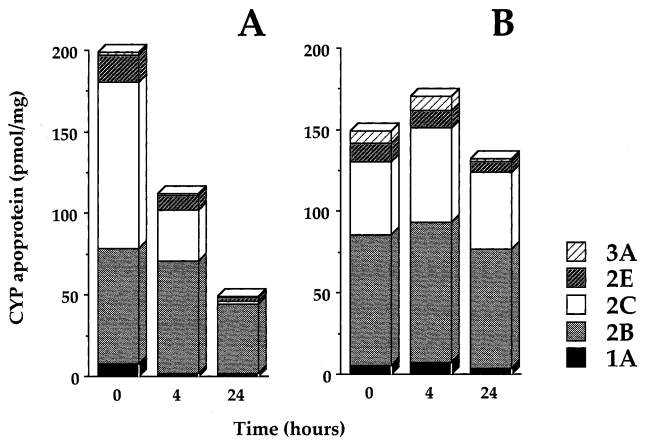

Fig. 2. CYP apoprotein pattern in standard (A) versus L-NAME-treated (B) cultures. Due to the high variability of the initial CYP content among different animals (see Table 1), the evolution in one representative culture is shown. Initial P450 holoenzyme content was 380.6 ± 42.6 and 298.0 ± 10.3 pmol/mg protein for standard and L-NAME-treated cells, respectively.

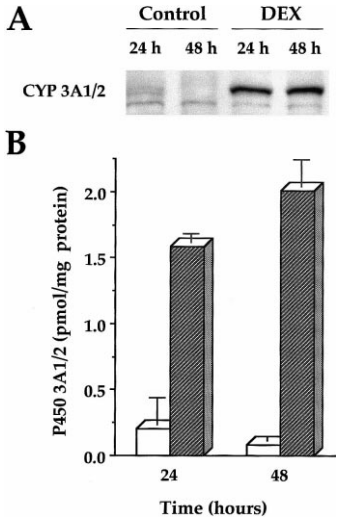

Fig. 3. DEX induction of CYP3A1/2 expression in L-NAME-treated hepatocytes. Western blot analysis (A) and quantitative estimation of 3A1/2 apoprotein (B) were performed as described in Section 2. Control = clear bars; DEX-treated = shaded bars. The anti-CYP3A4/5 used readily cross-reacts with rat CYP3A1 and 3A2 apoproteins, as can be observed in control cultures. Results are representative from three different experiments.

P450 apoenzymes in L-NAME-treated cells does not significantly differ from the initial content (Table 1), with the exception of the CYP3A1/2 isoforms which are only preserved to about 40% of the initial content. Thus, the early inhibition of NO synthesis not only sustains the total initial P450 holoenzyme content [3] but also allows the expression in culture of a CYP apoprotein pattern similar to that of freshly isolated hepatocytes.

3.3. *Inducibility of CYP expression in L-NAME-treated rat hepatocyte cultures*

We have examined the ability of L-NAME-treated cultures to increase CYP3A and 1A apoprotein content in response to their respective model inducers DEX or BNF. In vitro exposure to 50 μM DEX (Fig. 3) readily increases immunodetectable CYP3A1/2. Immunoblot analysis shows that only the higher molecular weight 3A-like protein (i.e. rat liver 3A1) appears significantly induced by the glucocorticoid (Fig. 3A). Quantitative estimation (Fig. 3B) shows a clear and early response to DEX, which increases CYP3A apoprotein 8- and 25-fold the control levels by 24 and 48 h of culture, respectively. Exposure to 50 μM DEX also increases the total P450 holoenzyme detectable after 48 h of culture (ca. 145 ± 14% of the corresponding control). Treatment with 50 μM BNF (Fig. 4) significantly increases CYP1A1/2 apoprotein levels. There is an important relative increase of the 1A1 isoform (Fig. 4A) whose levels are almost undetectable in freshly isolated cells (less than 10% of 1A2). Induction of CYP1A1/2 (Fig. 4B) is already very important after only 24 h of culture (34-fold the apoprotein content of the corresponding control) increasing further (70-fold the control content) after 48 h, at which it still does not seem maximal. BNF-induced expression of both isoforms actually reflects de novo protein synthesis since after 48 h of culture, 1A1/2 concentration in BNF-treated cells is significantly higher (ca. 10-fold) than the 0-time levels. In addition, exposure to BNF contributes to a net increase in the spectrally detectable P450 (ca. 210 ± 20 pmol/mg protein by 48 h as compared to 123 ± 9 pmol/mg protein in the corresponding control). If the estimates of the apoprotein content could be related to total spectrally discernible P450 holoenzyme, CYP1A1/2 would have increased from about 1% in freshly isolated cells to more than 10% of the total after 48 h of culture following in vitro exposure to BNF.

4. Discussion

Taken collectively, results from this and previous studies [3,4] support the view that the early loss of P450 haemoproteins in culture is the ultimate consequence of a NO-driven process spontaneously activated during the collagenase-based hepatocyte isolation procedure, which leads to accelerated and

Table 1
Estimation of the individual CTY apoprotein content in rat hepatocytes primary cultures (pmol/mg)

1A1/2		2B1/2	2C11	2E1	3A1/2
Standard cultures:					
0 h	3.1 ± 1.4	56.6 ± 11.4	66.0 ± 17.4	9.9 ± 1.1	2.0 ± 0.6
24 h	0.6 ± 0.3*	19.0 ± 4.4**	0.6 ± 0.1***	1.2 ± 0.2***	0.4 ± 0.1**
L-NAME-treated:					
0 h	2.1 ± 1.2	61.7 ± 37.2	67.8 ± 6.0	7.0 ± 1.5	3.5 ± 0.7
24 h	0.9 ± 0.5	60.0 ± 23.5	62.7 ± 13.8	4.4 ± 1.4	1.3 ± 0.3*

Data are means ± S.D. ($n = 3$ different cultures). Initial (time 0) content does not differ significantly in standard and L-NAME-treated cultures. Significant decrease relative to the corresponding 0 h value: *$P < 0.05$; **$P < 0.01$, ***$P < 0.001$.

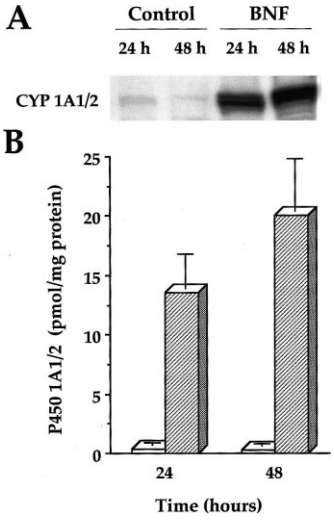

Fig. 4. BNF induction of CYP1A1/2 expression in L-NAME-treated hepatocytes. Western blot analysis (A) and quantitative estimation of 1A1/2 apoprotein content (B) were performed as described in Section 2. Control = clear bars; BNF-treated = shaded bars. The anti-CYP1A2 antiserum used in these experiments cross-reacts with both 1A1 and 1A2 apoproteins and therefore can be readily identified. Results are representative from three different experiments.

selective degradation of CYP apoproteins. Interestingly, constitutively expressed CYPs appear not equally affected. CYP1A2, 2C11 and 3A1/2 apoproteins decrease to very low levels after only 24 h of culture, while the 2B1/2 and 2E1 isoforms are better preserved. Quantitative estimations of the individual CYP isoform concentration further confirm that in conventional cultures rapid loss of total spectrally detectable P450 [3] is accompanied by a very significant change in the CYP apoprotein profile. Inhibition of NO synthesis (L-NAME-treated cells) (i) allows maintenance of the initial P450 levels in short-term culture [3], (ii) prevents the accelerated CYP apoprotein degradation observed in standard conditions and (iii) the initial constitutive CYP apoprotein pattern is satisfactorily preserved after 24 h of culture.

Different studies confirm that P450 enzymes are significant targets for NO. Through haem-nitrosylation (often followed by haem loss and degradation), *S*-nitrosylation and/or tyrosine nitration, NO is known to cause the irreversible loss of carbon monoxide discernible P450 content and activity [6–10]. NO can also inhibit the synthesis of new P450 molecules by decreasing CYP gene transcription and mRNA translation [13–16]. In short-term cultured hepatocytes, and in the absence of any other specific signal, the occurrence of a NO-dependent structural modification that labilises and directs CYP apoproteins to degradation is the most likely mechanism. The differential in vitro stability appears thus directly related to a differential sensitivity of the pre-existing enzymes towards NO or NO-derived species, more likely PN [4], generated within the early culture hours. Particularly remarkable is that the levels of CYP2C11 apoprotein, already decreased after only 4 h of culture and almost undetectable after 24 h, are fully preserved if only NO synthesis is suppressed. CYP2C11 is known to be unusually prone to haem loss and subsequent proteolytic disposal as compared to other CYP isoforms [17,18]. Also, CYP2C11 is particularly sensitive to inactivation through *S*-nitrosylation [8]. Both mechanisms could mediate NO interaction with CYP2C11 in standard hepatocyte cultures.

Difficulties in demonstrating P450 induction (in terms of protein or mRNA) in cultured hepatocytes are related to the well-known rapid postplating P450 decline process [2]. In this study we have confirmed that in L-NAME-treated cells, CYP1A1/2 and 3A1/2 enzymes can be significantly induced at very early culture times in response to their respective model inducers (BNF and DEX). The net increase in the apoprotein content is reflected by the very high induction factors that, particularly in the case of CYP1A1/2, exceed the values attained in most of the culture models available [2].

In conclusion, a rapid decline in CYP-dependent metabolism in culture appears to affect hepatocytes whatever the species or the culture system used [2]. Results in this study show that hepatocyte isolation and culture conditions also markedly influence the CYP isoform profile. It is generally recognised [2] that rat hepatocytes represent an extreme example of phenotypic instability in culture (if compared to hepatocytes from other species, i.e. human [19]). Thus, evidence of satisfactory maintenance of P450 in rat cells would bode well for hepatocytes from other species. Differences in the relative pattern of P450 loss should be caused by a different behaviour on NO (or PN) formation, likely related to qualitative differences in the hepatocyte isolation and culture protocols. If NO synthesis is fully blocked, conventional monolayer culture may represent the simplest alternative model for in vitro assay of drug metabolism. In the light of this and previous work [3,4], it appears that cNOS-derived NO may play a direct role in the modulation of drug biotransformation capability in liver cells, as first suggested for inflammatory conditions [9,20].

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