Endogenous nitric oxide is responsible for the early loss of P450 in cultured rat hepatocytes

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Abstract Loss of P450 during the early hours of monolayer formation is known to be the more serious limitation of primary cultured hepatocytes as an adequate model for the study of drug metabolism, toxicity and P450 induction. This study reports that endogenous nitric oxide (NO) formation is activated shortly after isolation by the classical collagenase-based liver perfusion methods. Both rapid P450 loss and aerobic mitochondrial energy metabolism impairment – with subsequent changes on glucose metabolism – are directly related to the high local generation of the radical at this stage. These effects can be reverted by the sole addition of NO biosynthesis inhibitors during liver perfusion and early culture hours, which allows catalytically active P450 to be preserved at levels close to those of the intact liver.

Key words: Hepatocyte primary culture; Drug metabolism; Nitric oxide; P450 content; Glycogenolysis; Glycolysis; AMP

1. Introduction

Rapid loss of P450 during the first hours of hepatocyte culture has for a long time been identified as the main limitation of this model for the study of drug biotransformation, toxicity and P450 induction. The early and quick decay of P450 is not an artifact nor reflects a generalized loss of the adult hepatocyte phenotype [1–3], appearing more an adaptative response to the in vitro culture environment. Accordingly (for review see [3]), the main approaches to improve maintenance of P450 protein and activity in rat liver cell cultures have pointed to redefinition of the culture medium (addition of nutrients, hormones, antioxidants, haem synthesis precursors, P450 ligands or inducers), culture support modification as well as the use of different coculture systems [4,5]. However, it appears that in cultured cells P450 can hardly be preserved to the level found in the intact liver: by 24 h in standard culture conditions, hepatocytes have already lost 80–90% of their initial P450 content, and only about 50% of the liver in vivo P450 can be preserved in the best conditions described (i.e. early exposure to specific P450 inducers or coculture). The current view is that P450 loss may well be the result of enhanced (and generalized) hemoprotein degradation at this stage of culture together with a failure of hepatocytes to re-synthesize P450 at adequate rates [3]; but the mechanism underlying these effects still remains poorly defined.

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Abbreviations: NO, nitric oxide; iNOS, inducible nitric oxide synthase; L-NAME, N^G-L-arginine methyl ester; CYP, cytochrome P450; TA, tienilic acid

In a previous article [6] we reported that other significant metabolic changes consistently occur in rat hepatocytes soon after cell plating: high glycolytic rates together with a rapid switch to glycogenolysis, a metabolic pattern reflecting that of the liver cells under hypoxia. Hence, this study was conducted to further characterize the mechanism involved in rat hepatocyte phenotypic changes at the stages of monolayer development, aiming to define better conditions to improve drug-metabolizing system expression in culture. Results show that massive nitric oxide (NO) formation is activated upon hepatocyte isolation through the classical collagenase-based method. NO generation represents in fact a key factor to explain both the quick P450 loss and the aerobic mitochondrial function impairment that ultimately explains glucose metabolism changes resembling hypoxia. Early loss of P450 in rat hepatocyte cultures can thus simply be avoided by the sole addition of NO biosynthesis inhibitors during liver perfusion and/or the early culture hours which allows preservation of catalytically active P450 levels close to those of the intact liver.

2. Materials and methods

2.1. Cell isolation and culture

Hepatocytes were isolated from fed male Sprague-Dawley rats (180–250 g) by reversed liver perfusion with collagenase (Boehringer Mannheim) and cultured on fibronectin-coated dishes in a 5% CO_2 humidified atmosphere, as described [6,7]. Culture medium was Ham's F12 (containing 10 mM glucose and 1 mM arginine) supplemented with antibiotics, 0.2% bovine serum albumin, 10^{-8} M insulin and 2% calf serum (Gibco). NO biosynthesis inhibitors were also added as indicated. Unattached cells were removed by changing the medium 1 h after plating. Cell viability (cell suspensions and monolayers) was assessed by the trypan blue dye exclusion test and always exceeded 95% in the experiments reported.

2.2. Determination of P450 content and activity

Total P450 was assayed [8] in microsomes from rat liver samples, freshly isolated cells or hepatocyte monolayers frozen under liquid N_2 (stored at −80°C). Microsomal fractions were obtained by centrifugation (100 000 × *g* for 90 min) of the S9 fractions from crude homogenates previously obtained through sonication (5–10 mg cell protein/ml in ice-cold 0.1 M potassium phosphate/1 mM EDTA, pH 7.4). Microsomal pellets were gently resuspended in a 50 mM Tris-HCl/20% glycerol buffer (pH 7.4) and stored at −20°C until P450 content and activity determination. CYP 2C11 activity was assayed as the rate of tienilic (TA) 5-hydroxylation in conditions of maximal initial rate, as described previously [9,10]. Protein content (total cellular or microsomal fractions) was determined by the Lowry procedure.

2.3. Determination of metabolites

Glycogen, ATP, ADP, AMP content and glycolytic activity (as lactate released to the medium) were determined by standard procedures as previously detailed [6]. Glycogen content of monolayers was also visualized cytochemically as PAS-positive material [11]. NO_2^- and nitrites ($NO_2^- + NO_3^-$) in the medium were determined by chemiluminescence [12].

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2.4. Statistical analysis

Experiments were performed in at least 3 different cultures with 3–4 plates per variable. Statistical analysis was done by the Student's *t*-test.

3. Results

Experiments were performed in very simple culture conditions, expressly avoiding supplementation with hormones and/or effectors, or with complex culture substrata, known to contribute to a better preservation of P450 in cultured liver cells.

3.1. Metabolic pattern of rat hepatocytes at the early stages of culture

Fig. 1A shows the quick and early P450 loss that characterizes rat hepatocytes upon culture in routine basal conditions; 6 h after cell plating, P450 content represents less than 50% of initial and by 24 h total P450 levels are less than 20% of that determined in the intact liver. In this same period, as shown (Fig. 1B), hepatocytes also exhibit high glycolytic rates (14–30 nmol lactate/min/mg cell protein for the first 3–4 h, depending on the culture) that are close to the maximal theoretical glycolytic flux as estimated by Hue [13]. Lactate in the culture medium stabilizes at around 2 mM, leading to significant acidification. Glycogenolysis is also unexpectedly activated soon after cell plating (Fig. 1C) in spite of the fact that hepatocytes are isolated from fed animals and cultured without exposure to glycogen phosphorylase-activating hormones. Glycogen depletion was maximal during the first 6 h and – after a transient insulin effect – proceeded at similar rates whether insulin was present or not (4–5 nmol glucose/min/mg cell protein). Thus, by 24 h of culture glycogen stores of hepatocytes (50–200 nmol glucose/mg cell protein) reflect those of livers from fasted rats. Analysis of the cell energy status at this stage shows (Fig. 1D) that AMP is also sharply increasing as a function of time so that by only 3 h it reaches values 4-fold higher than initial, decreasing gradually thereafter. Early changes in the AMP content (a major signal of anoxia) parallel early changes in the adenine nucleotide content (Fig. 1D). ATP is relatively stable (or slightly diminishes) but ADP levels significantly increase by only 2 h of culture; consequently, the ATP/ADP ratio of hepatocytes significantly decreases in short-term culture from 4.3 (in freshly isolated cells) to 2.4 as determined by 5 h of culture. These changes are, however, reversible (not shown) and 24 h later, lactate production rates, ATP, ADP and AMP levels have returned to basal values while loss of glycogen and P450 is irreversible.

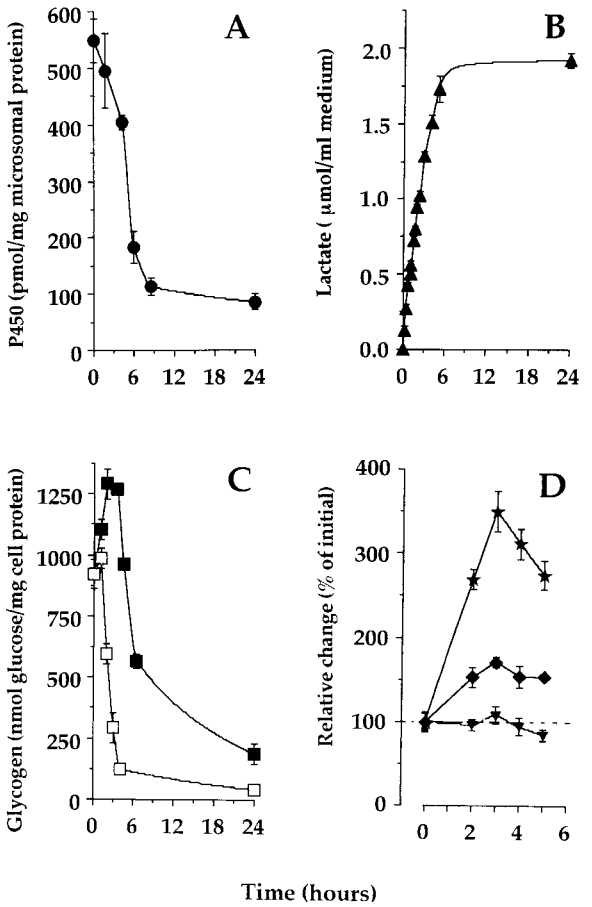

Fig. 1. Evolution of P450 (A), lactate production (B), glycogen (C) and adenine nucleotides (D) in short-term culture. Hepatocytes were cultured in standard medium containing (closed symbols) or not (open circles, C) 10^{-8} M insulin. P450 (A: ●), glycogen (C: ■,□) and adenine nucleotides (D: ATP ▾, ADP ◆ and AMP *) were assayed in cell fractions while glycolysis (B: ▲) was estimated as lactate accumulation in the medium. Initial values (100%) for ATP, ADP and AMP are 11.0 ± 1.1, 2.55 ± 0.25 and 0.17 ± 0.02 nmol/mg cell protein, respectively. Results are the mean ± S.E.M., $n = 4$ different cultures.

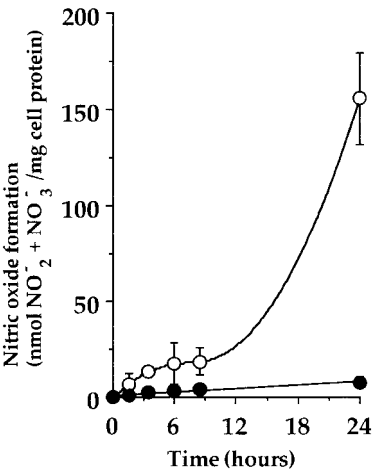

Fig. 2. Time-course of endogenous NO formation. Cells were cultured in standard medium containing (●) or not (○) 1 mM L-NAME that was added with the first medium renewal (1 h after plating). Data are the mean ± S.D. of 4 dishes from one representative culture out of three.

3.2. Activation of NO formation in short-term cultures

NO production (indicated by the accumulation of nitrite in the medium) is activated soon after hepatocyte plating in spite of the fact that cells are isolated from control animals uninduced for inducible nitric oxide synthase (iNOS) (Fig. 2). Thus, by 24 h of culture nitrite in the medium is about 50 μM. Ham's F12 medium does contain 1 mM L-arginine, well above the K_m for arginine (5–10 μM) as described for all the characterized NOS isoforms [14]. This was considered when NOS inhibitors were included in the medium. NO formation could be thus efficiently blocked by the addition of adequate amounts of known NO biosynthesis inhibitors, like N^G-L-arginine methyl ester (L-NAME) (Fig. 2).

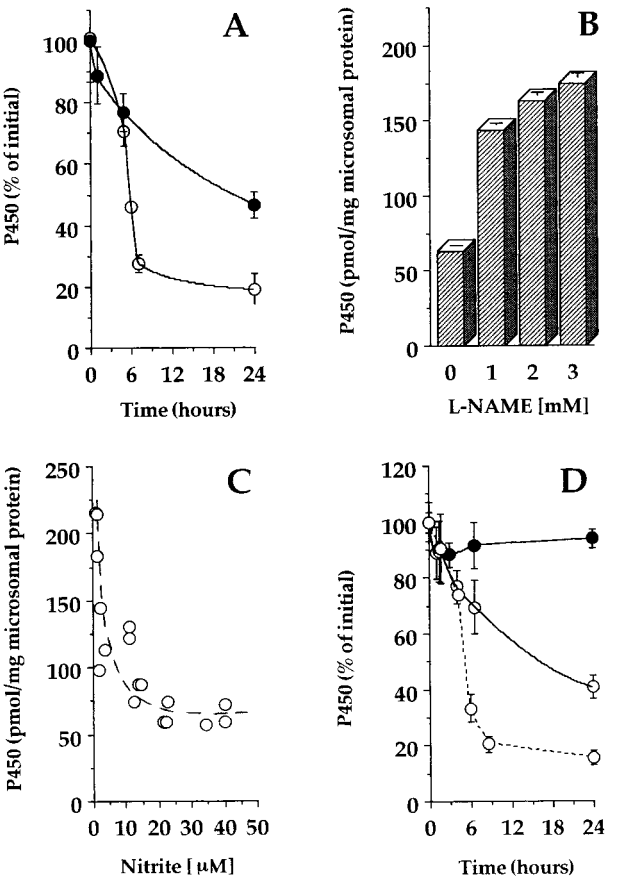

Fig. 3. Effect of the inhibition of NO biosynthesis on total P450 content of hepatocytes in short-term culture. A: Time-course of P450 levels in hepatocytes isolated in standard conditions and cultured in the presence (●) or absence (○) of 1 mM L-NAME. The inhibitor was added with the first medium renewal (1 h after plating). Data are mean ± S.D. of 4 dishes from one out of three similar experiments. 100% value (P450 content in freshly isolated cells) is 317.8 ± 17.6 pmol/mg microsomal protein. B: Dose-dependent effect of L-NAME on P450 recovery by 24 h of culture. Experimental conditions were equivalent to A, except that different L-NAME concentrations were used. Statistical significance was $P < 0.001$ for the three L-NAME variables vs. control (0 mM L-NAME), and $P < 0.01$ for 1 mM vs. 2 mM L-NAME. C: Correlation of microsomal P450 content to endogenous NO formation (indicated by $[NO_2^-]$ in the medium). Both were determined in parallel for the same plates in hepatocytes cultured for different times in the presence of variable amounts of L-NAME. Data correspond to four different cultures. D: Time-course evolution of total P450 in hepatocytes isolated in the presence of 1 mM L-NAME and subsequently cultured with (●) or without (○) 1 mM L-NAME. Data are mean ± S.D. of 4 dishes from one out of three similar experiments. 100% value is 437.8 ± 16.2 pmol/mg microsomal protein.The evolution of P450 in standard cultures (no L-NAME added) is over-imposed (dotted line, open circles) for an easy comparison.

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3.3. *Activation of NO production is directly involved in the early P450 loss and the metabolic impairment of hepatocytes observed in short-term cultures*

Inhibition of NO biosynthesis through addition of 1 mM L-NAME to the culture medium efficiently blocks the quick decay of P450 content (Fig. 3A). This results in a dose-dependent increase of the P450 level determined after 24 h of culture in the presence of increasing amounts of L-NAME (Fig. 3B). Correlation between P450 levels and NO_2^- concentration in the medium showed a logarithmic pattern (Fig. 3C) where maximal decrease of P450 is achieved when nitrite in the medium reaches a mean value of about 10 μM (already found by 6 h of culture in standard conditions).

Activation of endogenous NO synthesis seems to be a very early event upon cell isolation since inhibition of NO formation during only the liver perfusion stage (Fig. 3D) is enough to partially prevent the early P450 loss showed by control cultures and allows hepatocytes to retain more than 40% of their initial P450 by 24 h of culture. Best results are, however, obtained when 1 mM L-NAME is also present as a component of the culture medium for the first 24 h, which results in

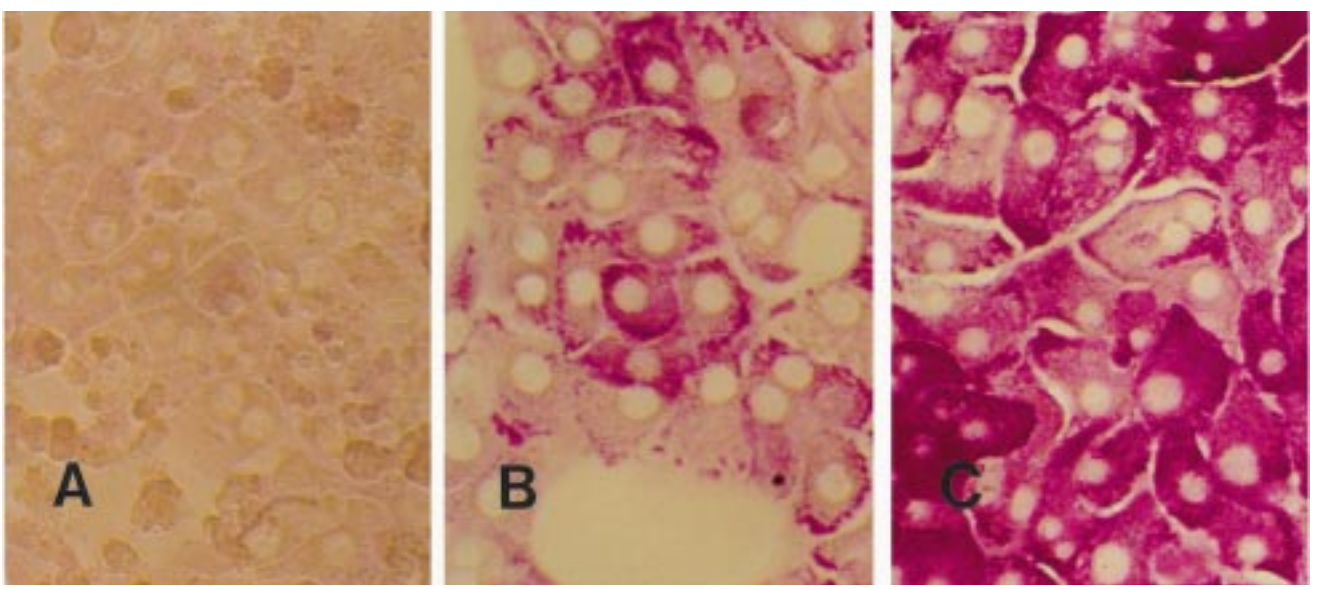

Fig. 4. Glycogen content of hepatocytes by 24 h of culture (PAS-positive material, magnification 400×). A: Cells isolated and cultured in routine conditions (see Section 2). B: Hepatocytes were isolated in medium containing 1 mM L-NAME and subsequently cultured in standard medium with no inhibitor added. C: Cells were isolated and cultured in the presence of 1 mM L-NAME. Glycogen levels correspond to a mean value of 52.2 ± 1.2 (A), 396.7 ± 19.8 (B) and 1250.1 ± 177.2 (C) nmol glucose/mg cell protein.

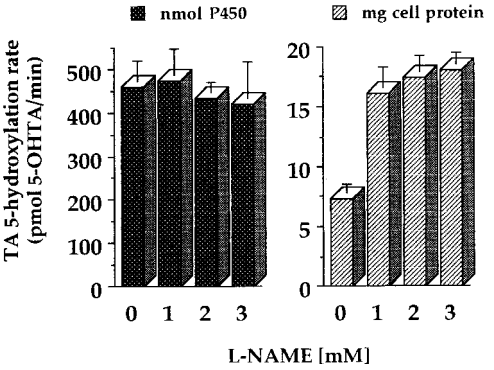

Fig. 5. Tienilic acid 5-hydroxylation rates by rat hepatocytes after 24 h of culture in the presence of variable amounts of L-NAME. Data are expressed per nmol P450 or per mg cell protein as indicated, and correspond to mean ± S.D. of 3 dishes from one representative experiment. Initial rates (freshly isolated hepatocytes, $n = 3$ different preparations) are: 396.2 ± 56.7 pmol 5-OHTA/min/nmol P450 and 26.5 ± 15.0 pmol 5-OHTA/min/mg cell protein.

the almost complete preservation of initial P450 levels. Efficient suppression of NO synthesis during isolation and short-term culture also significantly improves cell aerobic mitochondrial function, ultimately indicated by (i) a full preservation of the initial glycogen stores of hepatocytes (Fig. 4), and (ii) a significant decrease (by a half) of the glycolytic rates initially determined in standard conditions (not shown).

3.4. Culture survival and maintenance of the P450 holoenzyme and activity on exposure to L-NAME

Exposure to L-arginine analogs (i.e. L-NAME up to 3 mM) does not alter culture survival but rather improves it. This is reflected by monolayer development and morphology (Fig. 4) and hepatocyte viability and survival at this critical stage of culture (protein recovered by 24 h of culture is 90% of the initial inoculum vs. 75% in standard conditions). Preservation of total P450 is not an artifact simply due to data expression since the microsomal protein/cell protein ratio in cultures exposed for different times at various L-NAME concentrations (0.262, $r = 0.961$) does not differ from the ratio obtained in standard cultures (0.257, $r = 0.959$). Also, 24-h exposure to L-NAME does not modify the spectral characteristics of the P450 holoenzyme recovered (no apparent conversion to P420) that in these conditions is also catalytically fully active. TA 5-hydroxylase activity was tested as 2C11-dependent P450 activity (a major isoform in male rat liver). As can be observed (Fig. 5) TA hydroxylation rates determined in hepatocytes after 24 h of culture in medium supplemented with L-NAME (= 1 mM) are close to those obtained in freshly isolated cells when either referred per nmol P450 or per mg cell protein.

4. Discussion

Arginine-derived NO is a highly versatile signalling molecule involved in the regulation of a variety of cellular functions in different mammalian cell types (for review see [15–17]). Hemoproteins and proteins containing non-haem iron or iron-sulfur complexes are key molecular targets for the radical within the cells. Its lipophilic nature together with a very short half-life explains the special susceptibility of membrane-bound enzymes to locally generated NO. P450, a major class of haem-containing proteins in liver cells, has also been identified as a main target for NO action. As demonstrated in diverse cellular and subcellular systems exposed to either exogenous or cell-generated NO [18–21], irreversible loss of P450 involves haem-nitrosylation followed by haem loss and degradation which leads to significant decrease of P450 content and activity. In liver cells, NO was also shown to inhibit three iron-containing mitochondrial enzymes (aconitase of the citric-acid cycle and complexes I and II of the electron transport chain), thus impairing mitochondrial function [22,23]. NO also inactivates glyceraldehyde-3-phosphate dehydrogenase [24], which may lead to inhibition of the glycolytic/gluconeogenic pathways. Though liver apparently lacks significant constitutive NOS activity, both hepatocytes and non-parenchymal cells (Kupffer and endothelial) can be induced for iNOS activity [17,25,26]. In fact, different studies implicate NO as a key signal on the alterations of glucose homeostasis (i.e. inhibition of liver gluconeogenesis [27,28] and stimulation of glycogenolysis [29]) and on the generalized suppression of hepatic drug metabolism [19] associated with endotoxemia, septic shock and inflammation. Studies on hepatocytes from different species including humans [30–34] point to a direct role of hepatocyte-derived NO as a mediator of the cytokine-induced down-regulation of both expression and activitiy of specific CYP forms. In these conditions, NO impairs hepatocyte P450 function by irreversible destruction of the preexisting enzymes [18–21] but also inhibits the synthesis of new P450 molecules by decreasing CYP genes transcription and mRNA translation of either constitutive or inducible P450 isoforms [30,32–34].

Results here reported clearly demonstrate that unstimulated parenchymal cells isolated from control animals spontaneously produce high amounts of NO. NO synthesis parallels the irreversible loss of total P450 and also the impairment of the aerobic mitochondrial/glucose metabolism consistently observed at this stage. All of these effects can be easily explained by NO interaction with liver iron-containing enzymes. Generation of NO is clearly dependent on hepatocyte NOS activity, since addition of NOS inhibitors efficiently blocks NO formation. A direct relation of NO to the phenotypic changes described is also demonstrated by the fact that (i) loss of P450 correlates well with NO_2^- accumulation, with a maximal deleterious effect observed when nitrite in the medium reached about 10 μM, a physiologically significant level, that is already achieved by 6–8 h of culture; (ii) metabolic impairment of hepatocytes is also ascribed to this same period, i.e. the stages of monolayer development; and (iii) inhibition of NO synthesis is enough to completely prevent both early P450 loss and cell energy metabolism alteration (ultimately reflected by complete glycogen depletion) in culture.

Hepatocyte endogenous NOS activity is not merely a cell culture artifact since the level of nitrites in the medium (149.1 ± 23.5 nmol/mg cell protein after the first 24 h) is very significant and in the range of those produced by hepatocytes from different species following iNOS induction with endotoxin or proinflammatory cytokines (60–350 nmol/mg cell protein [26,30,31] by 24 h of exposure, depending on the study). Studies on the role of cytokines on hepatocyte iNOS induction are, however, often performed after an initial 24-h culture period needed for metabolic 'stabilization' of freshly isolated cells. Interestingly, the few studies ascribed to the first 24 culture hours [35–37] show a significant basal

NOS activity (with nitrites in the medium reaching 40–107 nmol/mg cell protein) as compared to basal activity in the second 24–48-h period (6–18 nmol nitrites/mg cell protein [26,30,31] or 8 nmol/mg cell protein for this study, not shown). Whether NO synthesis results from constitutive NOS or the activity is due to a low level of induction (as suggests the time-course accumulation of nitrites in the medium) remains to be elucidated. Nevertheless, our results indicate that activation of NO synthesis is a very early event inherent to hepatocyte isolation and culture in standard conditions. Other signals (apart from cytokines) should be invariably present during the collagenase perfusion or the early culture steps and may also play a role as stimuli for either NOS activation or iNOS induction. This may include contaminants of commercial collagenases, intra-extracellular calcium movements (as Ca^{2+}-chelation and replacement is a part of the isolation procedure), oxidative stress [38] or even peptides derived from extracellular matrix components (laminin, collagen, fibronectin) recently described as efficient inducers of iNOS in human lymphomononuclear cells [39].

In conclusion, this study establishes that accelerated P450 degradation in rat hepatocyte primary cultures is directly related to endogenous NO overproduction, systematically detected at the stages of monolayer development in standard cultures. Supplementation of perfusion and culture media with inhibitors of NO biosynthesis thus provides a very simple method to avoid this phenomenon. As arginine supply plays a crucial role on the post-transcriptional regulation of NOS activity [15–17], concentration of L-arginine in the culture – which varies considerably depending on the medium, i.e. from 0.2 mM for Williams E to 2.9 mM for Leibowitz L15 – should be considered when establishing the concentration of the inhibitor to be added. This should be high enough to efficiently block NO formation without affecting arginine requirements for protein, amino acid and urea synthesis. In our culture conditions 1 mM L-NAME (also 1 mM aminoguanidine, not shown), although not sufficient for complete suppression of NO biosynthesis, seems enough for full preservation of P450 to levels equivalent to those of the intact liver. After 24 h of in vitro exposure to the inhibitor, the P450 recovered is catalytically fully active and we did not detect any cellular damage (in fact exposure to L-NAME ameliorated cell monolayer development and morphology). The mechanism underlying NO interaction with P450 suggests that NO is a non-specific inactivator of these hemoproteins but, through differences in haem accessibility, NO may well differentially affect constitutive or inducible P450 isoforms. Our study focused on constitutively expressed P450 2C11. Studies aimed to evaluate if NO synthesis inhibition does maintain the relative P450 pattern of the intact liver (from either control or induced rats) in culture are anticipated.

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