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Role of temperature on protein and mRNA cytochrome P450 3A (CYP3A) isozymes expression and midazolam oxidation by cultured rat precision-cut liver slices

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Abstract

The cytochrome P450 3A (CYP3A)-mediated midazolam oxidation was studied in rat precision-cut liver slices (PCLS) maintained for 20 hr at 4, 20 and 37°, and further incubated for 8 hr at 37°. Either at 4 or 20°, midazolam was oxidised by PCLS at similar rates to that observed in freshly cut slices. Moreover, PCLS kept a regioselectivity since 4-hydroxylation was more important than 1′-hydroxylation. Conversely, PCLS totally lost their capacity to oxidise midazolam after 20 hr at 37°, and both CYP3A2 protein and mRNA were not detected. CYP3A1 protein was unaffected by a temperature of 37° but its mRNA was totally lost. By blocking transcription with actinomycin D, the decay of both CYP3A mRNAs followed the same profile at either 20 or 37°, indicating that temperature affected the CYP3A2 protein stability. Cell functionality was not involved in such an impairment since the low values of ATP, GSH and protein synthesis rates observed at 4 and 20° were rapidly restored, when PCLS were further incubated at 37°. The use of rat supersomes expressing either CYP3A1 or CYP3A2, strongly supported the hypothesis that 4-hydroxymidazolam was mainly formed by CYP3A2. These results suggest that: (1) CYP3A1 protein is constitutive and largely expressed in rat liver slices; (2) regioselective midazolam oxidation appears to be mainly CYP3A2 dependent; and (3) since CYP3A isoforms have similar half-lives (about 10–14 hr), the loss of CYP3A2 protein at 37° might be due to a selective targeting (phosphorylation ?) leading to proteolytic disposal by the proteasome.

Keywords: Liver slices; CYP3A; Midazolam oxidation; Phase I metabolism; Cold preservation

1. Introduction

Liver transplant patients receive high doses of multiple drugs, but little information is available about the effect of liver transplantation on drug biotransformation. Many factors, such as a decrease of hepatic energy status by cold storage or hormone release after surgical stress, could influence drug metabolism after liver transplantation [1]. Cold storage or hypothermic preservation is mainly characterised by many alterations including depletion of high-energy phosphates, intracellular acidosis, hypothermia-induced cell swelling and free-radical-induced cellular injury [2,3]. In particular, sinusoidal endothelial cells and Kupffer cells have been identified as major targets of hypoxia-reoxygenation injury [4,5]. Both xenobiotic and intermediary metabolism of hepatocytes may be influenced, therefore, by reactive intermediates and soluble factors which are released from such nonparenchymal cells [6,7].

These cellular interactions may have important pharmacological and toxicological consequences which require to be further explored. Nevertheless, cell cultures and co-cultures have essential disadvantages such as uncontrolled damage during cell isolation procedures, loss of tissue architecture and intercellular communication (inducing dedifferentiation), and lack of organ-specific composition including parenchymal and nonparenchymal cells. Some

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Abbreviations: CYP3A, cytochrome P450 3A; PCLS, precision-cut liver slices; RT-PCR, reverse transcriptase-polymerase chain reaction; WME, Williams'medium E.

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of these difficulties could be overcome by using the *in vitro* system of tissue slices because they contain all the cell types present in the whole organ *in vivo*, within their normal spatial relationships [8].

In view of the success with liver transplantation, we were interested to study the effects of cold preservation on cytochrome P450 and associated enzyme activities by using PCLS. Actually, the CYP3A is one of the most important cytochrome P450 subfamilies and plays a significant role in the metabolism of approximately half the drugs in use today [9]. The catalytic activity of CYP3A enzymes may be influenced by a wide array of compounds, age, diseases, and environmental factors. During organ transplantation, liver is preserved at cold temperature and then reperfused at 37°. Therefore, the influence of different maintenance temperatures (4, 20 and 37°) as well as a subsequent incubation at 37° on CYP3A-mediated oxidation by PCLS was studied. The metabolic ability of PCLS was estimated by recording the formation of both 1′- and 4-hydroxymidazolam, the two main metabolites of midazolam. This drug is a widely used anaesthetic, which is mainly metabolised by CYP3A isoforms [10]. Since in untreated animals, CYP3A1 has been reported to be either not expressed [11,12] or constitutively expressed [13,14], whereas CYP3A2 is the predominant expressed form in adult male rats [15,16], a particular attention was given to the activity of these CYP3A isoforms. Cell lysates of liver slices were analysed for their CYP3A1 and CYP3A2 apoprotein by Western blots and reverse transcriptase-polymerase chain reaction (RT-PCR) was used to evaluate the specific mRNA gene products corresponding to each of the above CYP3A isoforms. The maintenance of ATP and glutathione (GSH) content and the capacity to synthesise proteins were used as established markers to assess the functional capacity of liver slices.

2. Materials and methods

2.1. Animals

Male Wistar rats weighing 250–300 g were purchased from Iffa-Credo (Les Oncins-France) and housed in individual cages in a temperature- and light-controlled room (12 hr dark/light cycle). They received standard diet AO3 (U.A.R., Villemoisson-sur Orge) and water *ad libitum*.

2.2. Chemicals

Williams'medium E (WME) and fetal calf serum were purchased from Gibco BRL. Gentamicin sulfate, leupeptine, phenylmethylsulfonyl fluoride, antipain, dithiothreitol, EDTA, Triton X-100 were obtained from Sigma Chemicals. Midazolam (Dormicum^{R}) and flunitrazepam were obtained from Roche. L-Glutamine was obtained from ICN Biomedicals Inc, and insulin from Actrapid HM, Novo Nordisk. Rabbit antibody to rat CYP3A1 and CYP3A2, and goat anti-rabbit IgG HPR were purchased from Chemicon. Western immunoblotting kit employing enhanced chemiluminescence detection and polyvinyldifluoride membranes was obtained from Amersham. Specific primer pairs CYP3A1 and CYP3A2 were synthesised at the department of Hematology (Université Catholique de Louvain). Rat CYP3A1 and CYP3A2 supersomes were from Gentest Corporation. All other chemicals used were of the purest grade available.

2.3. Preparation and incubation of PCLS

Rat surgical procedures were carried out under pentobarbital (60 mg/kg) anaesthesia. PCLS (about 200–250 μm thickness) were prepared by using the Krumdieck tissue slicer. They were stored 30 min at 4° in WME containing 10% of fetal calf serum, glutamine (2 mM) and insulin (100 nM). Thereafter, PCLS were transferred to vials containing WME (1 slice/2 mL) supplemented with glutamine (2 mM), insulin (100 nM) and 50 μg/mL gentamicin sulfate. Vials were placed in a shaking water bath (80 oscillations/min) according to the different experimental conditions:

(1) incubation of PCLS for 8 hr at 37° under a continuous flow of O_2/CO_2 (95%:5%), hereafter referred to as "freshly cut slices";
(2) maintenance of PCLS for 20 hr at 37° under a continuous flow of O_2/CO_2 (95%:5%);
(3) maintenance of PCLS for 20 hr at 20° under a continuous flow of O_2/CO_2 (95%:5%);
(4) before the maintenance of PCLS at 4° for 20 hr, vials were saturated for 30 min with a mixture of O_2/CO_2 (95%:5%).

After 20 hr (conditions 2, 3 and 4), PCLS were rinsed in saline and further incubated for 8 hr in fresh WME as described above. The sampling, for all the four conditions, were performed at the beginning and at the end of such 8-hr incubation time.

2.4. ATP content

Liver slices were taken, washed twice in saline and sonicated immediately in 1 mL of 2% perchloric acid. The intracellular ATP content was measured on neutralized perchloric acid extracts using the ATP Bioluminescense Assay Kit CLS II from Boehringer-Mannheim (Germany). The results are expressed as nanomole of ATP per milligram of protein.

2.5. GSH content

Reduced GSH was assayed using a modified method of Hissin and Hilf [17]. PCLS were taken, washed twice in saline and sonicated in 1 mL of 6% tricloroacetic acid and centrifuged for 30 min at 3600 *g* at 4°. Aliquots of the

standards and precipitated PCLS were added to tubes containing phosphate/EDTA buffer (pH 8) and 1 mg/mL *o*-phtalaldehyde (200 μL), then stored in the dark (15 min). Fluorescence was measured (excitation 350 nm, emission 420 nm) using a spectrofluorimeter, Perkin-Elmer LC-50B. The results are expressed as nanomole of GSH per milligram of protein.

2.6. Protein synthesis

Protein synthesis was estimated by measuring the incorporation of [^{14}C]leucine (specific activity 94 μCi/mmol, 0.8 mM unlabeled leucine) into the protein pellet obtained by perchloric acid precipitation as described by Seglen [18]. At the indicated times, PCLS were rinsed in saline, placed in a fresh medium containing unlabeled leucine in the presence of [^{14}C]leucine, and the incorporation of radiolabeled leucine was measured during 2 hr. The results were expressed as dpm of leucine incorporated per milligram of protein per minute. The amount of protein was determined by the Lowry's method using bovine serum albumin as standard [19].

2.7. Midazolam metabolism

1′-Hydroxy- and 4-hydroxymidazolam were quantified by using an HPLC method described by Eeckhoudt *et al.* [20]. At the indicated times, PCLS were rinsed in saline, placed in a multiwell culture plate (2 mL/slice) in a medium supplemented with midazolam (0.125 mg/mL), and the formation of metabolites was recorded during 50 min. This period (50 min) ensures the linearity in the formation of the 1′-hydroxy- and 4-hydroxymidazolam (data not shown). Two millilitres of incubation medium were stored at −20° for further analysis. Three hundred microlitres of an aliquot of medium were treated with 1 mL of 0.3 M sodium acetate buffer (pH 5) containing 5000 IU/mL β-glucuronidase and 38 μL of the internal standard flunitrazepam (50 μg/mL). After incubation for 2 hr at 37°, 200 μL of 2% NaOH and 4.5 mL of cyclohexane–diethyl ether (31:69) were added and the mixture was extracted on a rotary mixer for 10 min at 4° and then centrifuged at 2000 *g* for 10 min at 4°. The organic phase was transferred to a clean tube and evaporated to dryness under a stream of nitrogen. The residue was dissolved in water–acetonitrile (95:5) and 20 μL were injected onto the HPLC column. The results were expressed as microgram of metabolites per milligram of protein.

2.8. Western immunoblotting

Liver slices were sonicated in Tris–HCl buffer (pH 7.4) containing phenylmethylsulfonyl fluoride (0.1 mM), leupeptine (2 μg/mL), antipain (2 μg/mL), dithiothreitol (2 mM), EDTA (0.1 mM) and Triton X-100 (0.4%). After centrifugation, the supernatant was stored at −20°. The proteins were subjected to sodium dodecylsulfate/polyacrylamide gel electrophoresis (SDS/PAGE) (10% separating gel) followed by electroblot to polyvinyldifluoride membranes. The membrane was blocked 1 hr at 25° in Tris–sodium buffer (pH 7.4) containing 5% (w/v) powdered milk protein followed by an incubation with diluted antibodies at 25° in a fresh solution of powdered milk protein (1% w/v) in Tris–sodium buffer. The membrane was washed and incubated with a dilution of secondary antibody at 25°. The primary and secondary antibodies were diluted respectively 1/16,000 and 1/10,000 for CYP3A1, 1/8,000 and 1/10,000 for CYP3A2. Immunodetection was performed using the ECL™ detection kit (Amersham).

2.9. Total RNA isolation and RT-PCR

Either at the beginning or at 4-hr incubation at 37°, PCLS were rinsed with sterile saline, frozen immediately in liquid nitrogen and stored at −80°. Total RNA was isolated from frozen liver slices (200 mg) according to Chomczynski and Sacchi [21] using the RNA agents Total RNA Isolation System (Promega). The estimation of mRNA CYP3A1 and CYP3A2 was perfomed by RT-PCR using Access RT-PCR System Kit (Promega). Oligonucleotide primers for CYP3A1 and CYP3A2 were chosen on the basis of the published cDNA sequences [22]. Specific primer pairs were as follows: CYP3A1, 5′-GATGTTGAAATCAATGGTGTGT-3′ and 5′-TTCAGAGGTATCTGTGTTTCC-3′; CYP3A2, 5′-AGTAGTGACGATTCCAACATAT-3′ and 5′-TCAGAGGTATCTGTGTTTCCT-3′. RTPCR produced a single fragment of 289 bp size for CYP3A1 and 252 bp for CYP3A2, in agreement with the literature [23]. RT-PCR reaction mixture (50 μL) contained 50 pmol of each primer, 0.2 μM each dNTP, 2 mM $MgSO_4$, 5 units AMV reverse transcriptase, 5 units TFL DNA polymerase and 0.983 μg sample RNA. The cycle number used for each primer set was adjusted to ensure linear amplification. Either 22 (CYP3A1) or 19 (CYP3A2) cycles, consisting of 94° for 30 s, 55° (CYP3A1) or 54° (CYP3A2) for 1 min, and 68° for 2 min, were run in a Perkin Elmer GeneAmp PCR System 2400 Thermal Controller. The last elongation step was extended to 7 min. Reaction products (3 μL), together with β-actin cDNA were electrophoresed on a 1.8% agarose gel in TAE buffer (40 mM Tris base, 5.7% acetic acid, 1 mM EDTA, pH 8), containing 0.1 mg/mL ethidium bromide. RT-PCR products were detected by using a UV transilluminator, gels were photographed and further scanned (Sharp JX 325 Scanner). The relative amounts of the bands was calculated by densitometry using the software Image Master™ (Pharmacia-LKB).

2.10. Statistical analysis

The results are expressed as mean ± standard error of the mean (SEM). Analysis of variance (two-way ANOVA

with the interaction time/treatment) was used to compare the respective parameters. For statistical comparison of results at a given time point, data were analyzed using Student's *t*-test. The level of significance was set at $P < 0.05$.

3. Results

At the beginning of the 8-hr incubation at 37°, the oxidation of midazolam by freshly cut slices yielded about twice more 4-hydroxymidazolam (Fig. 1A) than 1′-hydroxymidazolam (Fig. 1B). However, at the end of such an incubation time, they partly lost such a capacity, and the formation of 4-hydroxymidazolam decreased faster than 1′-hydroxymidazolam. A similar profile was seen in PCLS maintained for 20 hr at either 4 or 20° and further incubated for 8 hr at 37° in fresh WME. However, after a maintenance for 20 hr at 37°, the formation of both metabolites by PCLS was already decreased by about 80%, the regioselectivity was not retained (the 4-hydroxy/1′-hydroxy ratio was only 1.3, as compared to about 2.0 in the other conditions) and the further incubation for 8 hr at 37° in a fresh WME did not modify such a depressed metabolism.

Since enzyme activity is mainly dependent on the quantity of protein, the amount of CYP3A1 and CYP3A2 proteins was estimated by Western blots (Fig. 2). In order to do a more quantitative comparison, densitometry analysis was applied to spots of both CYP3A isoforms in PCLS samples and CYP3A isoforms in rat supersomes (Gentest). From that values, it was deduced a ratio between O.D. units of CYP3A isoform per milligram of proteins by lane. As compared to samples of freshly cut slices obtained at the beginning of the 8-hr incubation at 37° (lane 1), the amount of CYP3A1 protein of liver slices maintained for 20 hr remained fairly constant whatever the temperature, namely 37 (lane 3), 20 (lane 5) and 4° (lane 7). In addition, a subsequent incubation for 8 hr at 37° did not modify the amount of CYP3A1 protein either in freshly cut slices (lane 2) or in slices previously exposed for 20 hr to such different temperatures (lanes 4, 6 and 8). The amount of CYP3A2 protein was, however, dramatically affected by the temperature. As compared to samples of freshly cut slices obtained at the beginning of the 8-hr incubation at 37° (lane 1), CYP3A2 protein was no longer detected after 20 hr at 37° (lane 3). At 20° (lane 5), CYP3A2 protein was markedly decreased but it remained unchanged at 4° (lane 7). The subsequent incubation for 8 hr in fresh WME at 37° further decreased the CYP3A2 protein either in freshly cut slices (lane 2) or in slices maintained for 20 hr at 4° (lane 8). In slices previously maintained at 37 (lane 4) or 20° (lane 6), CYP3A2 protein was no longer detected.

The expression levels of CYP3A1 and CYP3A2 mRNAs were examined by RT-PCR (Fig. 3) and the O.D. units in each lane were corrected by the respective β-actin absorbance. The levels of CYP3A mRNAs remained fairly constant either in freshly cut slices at zero time (lanes 1) or in slices maintained for 20 hr at 20 (lanes 5) and 4° (lanes 7). Conversely, they were strongly decreased after a maintenance for 20 hr at 37° (lanes 3). When slices were further incubated at 37° for 4 hr, no dramatic changes were observed in both mRNAs from slices previously maintained for 20 hr at 20° (lanes 6) and 4° (lanes 8), but a strong decrease was seen in CYP3A2 mRNA from freshly cut slices (lane 2) while CYP3A1 mRNA was unaffected (lane 2). Moreover, in slices previously maintained at 37°, neither CYP3A1 nor CYP3A2 mRNA were detected (lanes 4).

A temperature of 37° is equally affecting both CYP3A mRNAs but only the CYP3A2 protein. Indeed, both mRNAs were not affected by a maintenance at either 20 or 4°, but CYP3A2 protein was no longer detected at 37° and it was strongly impaired at 20°. Therefore, the question about the CYP3A mRNA stability was raised. By blocking transcription with actinomycin D, the profile of mRNAs decay was followed for 12 hr at either 20 or 37° (Fig. 4). No dramatic differences were observed between the same isoform at different temperatures or different isoforms at the same temperature, indicating that mRNA degradation is unlikely the cause of CYP3A2 protein instability at 37°.

A potential loss of PCLS viability under 37° may not be evoked to explain their decreased capacity to oxidise midazolam. Actually, markers of viability and metabolic function, such as ATP content (Fig. 5A), GSH level (Fig. 5B) and protein synthesis rates (Fig. 5C), strongly recovered as soon as PCLS were further incubated for 8 hr at 37° in a fresh WME. Indeed, after a maintenance time of 20 hr at 37°, the values of GSH remained fairly constant but the ATP content and protein synthesis rates were significantly decreased by about 35–40% as compared to freshly cut slices. Decreasing the maintenance temperature from 37° to either 20 or 4°, caused a temperature-dependent inhibitory effect. However, when such PCLS were further incubated in fresh WME for 8 hr at 37°, a strong recovery in all these markers was observed, indicating that the inhibitory effect of low temperatures was totally reversible by the resumption of the metabolism.

Since the loss of midazolam oxidation may be explained by a decreased amount of CYP3A2, and the 4-hydroxymidazolam derivative appears to be less formed than 1′-hydroxymidazolam under 37°, a link between regioselectivity and isoform specificity was hypothesised. Table 1 shows the rates of midazolam metabolites formation by untreated rat liver microsomes, and rat supersomes (Gentest) either expressing CYP3A1 or CYP3A2 isozymes. Rat liver microsomes yielded about 2.8 times more 4-hydroxymidazolam than 1′-hydroxymidazolam, closely to that observed in freshly cut slices. When supersomes expressing CYP3A1 were incubated in the presence of

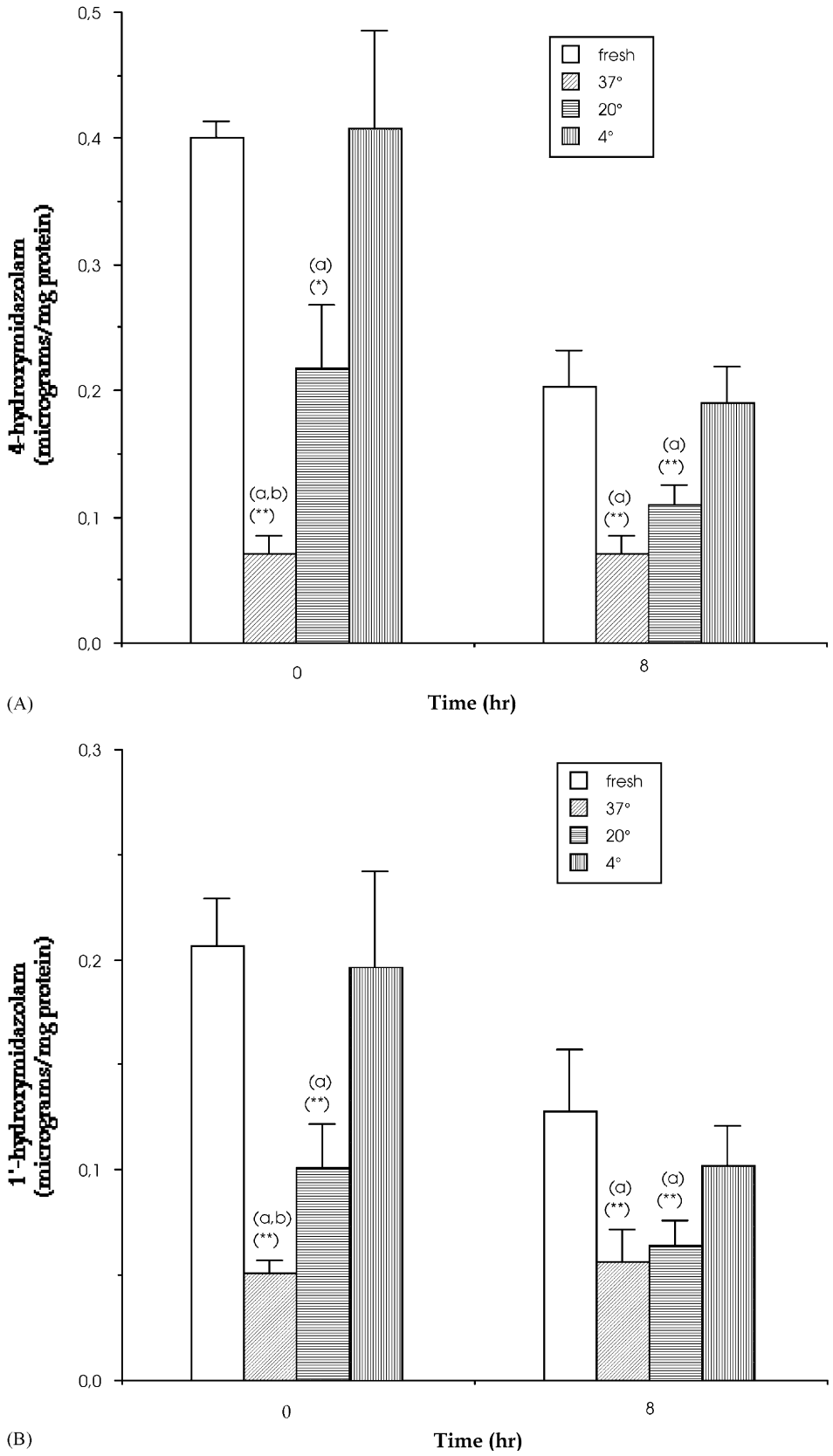


Fig. 1. The effect of temperature on midazolam oxidation by CYP3A. Liver slices were maintained for 20 hr at either 4, 20 or 37°. Afterwards the slices were rinsed in saline and incubated for 8 hr at 37° in a fresh WME. At the beginning and at the end of such an incubation time, slices were placed for 50 min in the presence of midazolam and the formation of 4-hydroxymidazolam (A) and 1′-hydroxymidazolam (B) was measured as described under Section 2. Experiments were performed in duplicate and values represent the mean ± SEM from at least three rats. The results were compared to freshly cut slices incubated for 8 hr at 37°. (*) $P < 0.05$ as compared to values in freshly cut slices; (**) $P < 0.05$ as compared to values in slices previously maintained at 37°; (***) $P < 0.05$ as compared to values in slices previously maintained at 20°.

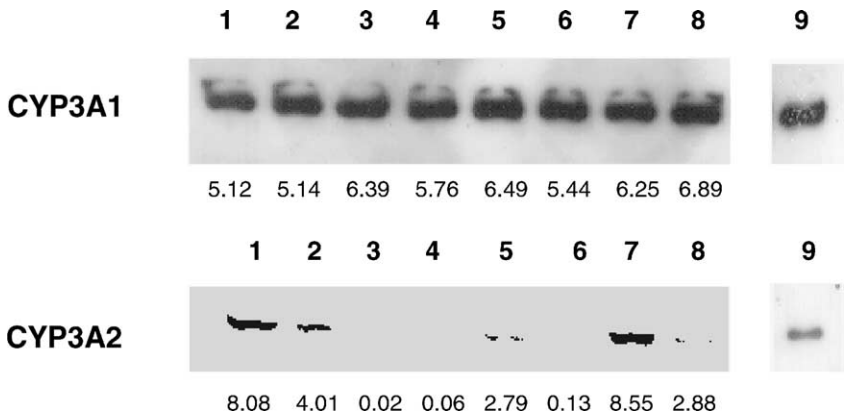


Fig. 2. The effect of temperature on CYP3A1 and CYP3A2 proteins. Liver slices were maintained for 20 hr at either 4, 20 or 37°. Afterwards the slices were rinsed in saline and incubated for 8 hr at 37° in a fresh WME. At the indicated times, slices were homogenized and immunoblots were performed as described under Section 2. The supernatants of slices (2.5 μg protein per lane for CYP3A1 and 10 μg protein per lane for CYP3A2) were prepared from freshly cut slices at the beginning (lane 1) or at the end of the incubation of 8 hr at 37° (lane 2); and at the beginning of the incubation of 8 hr at 37° from slices previously maintained for 20 hr at 37 (lane 3), 20° (lane 5) and 4° (lane 7). The lanes 4, 6 and 8 are corresponding to CYP3A1 and CYP3A2 proteins at the end of the incubation of 8 hr at 37° from slices previously maintained for 20 hr at 37, 20 and 4°, respectively. Lanes 9 correspond to rat supersomes expressing either CYP3A1 (26 pg) or CYP3A2 (48 pg). Values were obtained as follow: (slices CYP3A1/2 O.D./mg of protein divided by supersomes CYP3A1/2 O.D./mg of protein) × 1000.

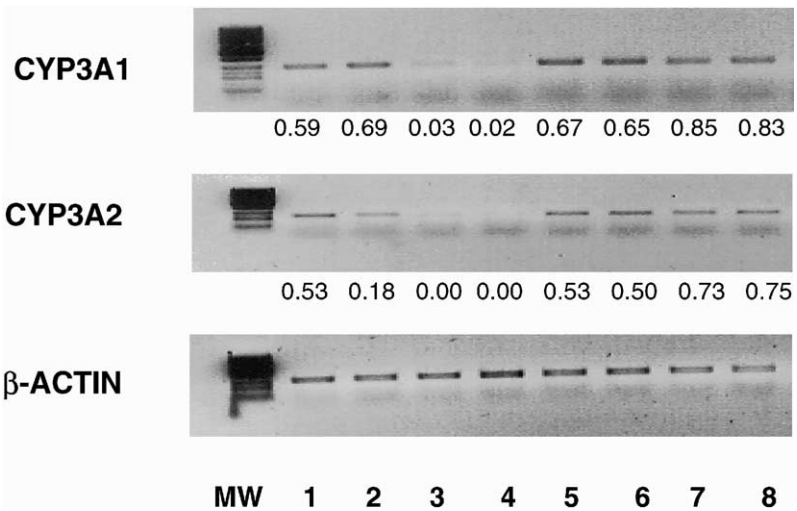


Fig. 3. The effect of temperature on CYP3A1, CYP3A2 and β-actin mRNAs. Liver slices were maintained for 20 hr at either 4, 20 or 37°. Afterwards, the slices were rinsed in saline and incubated for 4 hr at 37° in a fresh WME. At the indicated times, RNA isolation and RT-PCR were performed as described under Section 2. The CYP3A1, CYP3A2 and β-actin mRNA expression are shown from freshly cut slices at the beginning (lane 1) or at the end of 4 hr at 37° (lane 2); and at the beginning of 4-hr incubation at 37° from slices previously maintained for 20 hr at 37 (lane 3), 20 (lane 5) and 4° (lane 7). The lanes 4, 6 and 8 are corresponding to mRNA expression at the end of 4 hr at 37° from slices previously maintained at 37, 20 and 4°, respectively. Values were obtained by dividing O.D. units of CYP3A1/2 by O.D. units of β-actin.

Table 1
Formation of midazolam metabolites

	4-Hydroxymidazolam (μg metabolites formed/min/mg protein)	1′-Hydroxymidazolam (μg metabolites formed/min/mg protein)
Rat liver microsomes	0.297	0.105
Freshly cut liver slices	0.022	0.008
Rat CYP3A1 supersomes	0.063	0.062
Rat CYP3A2 supersomes	0.234	0.097

Samples were incubated for 30 (2 slices/mL) and 16 min (supersomes 0.21 mg protein/mL; microsomes 0.6 mg protein/mL) in the presence of 400 μM of midazolam. Aliquots of the suspension were taken and the formation of 4-hydroxymidazolam and 1′-hydroxymidazolam was measured as described under Section 2. Experiments were performed in duplicate and values represent the mean of two rats.

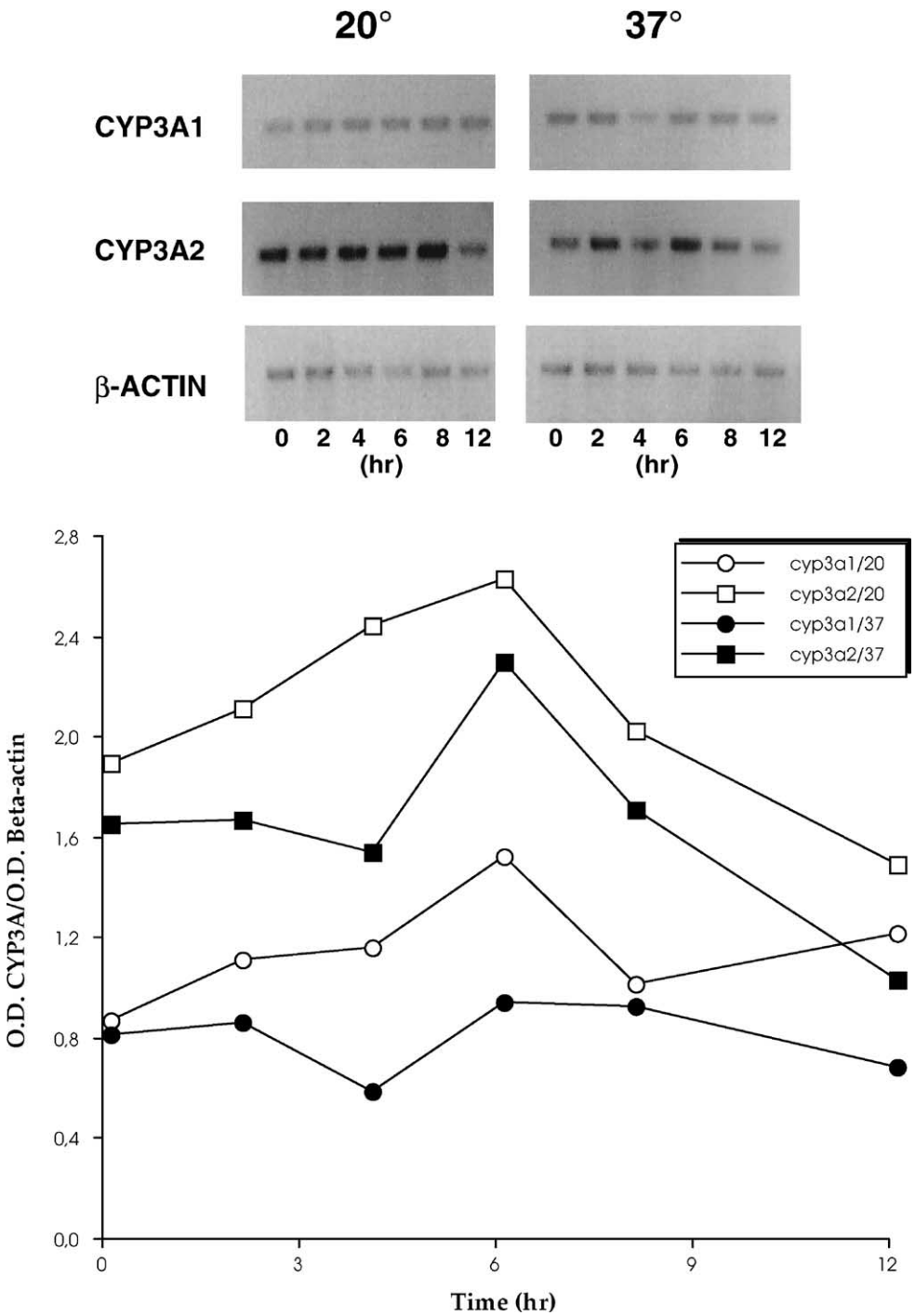
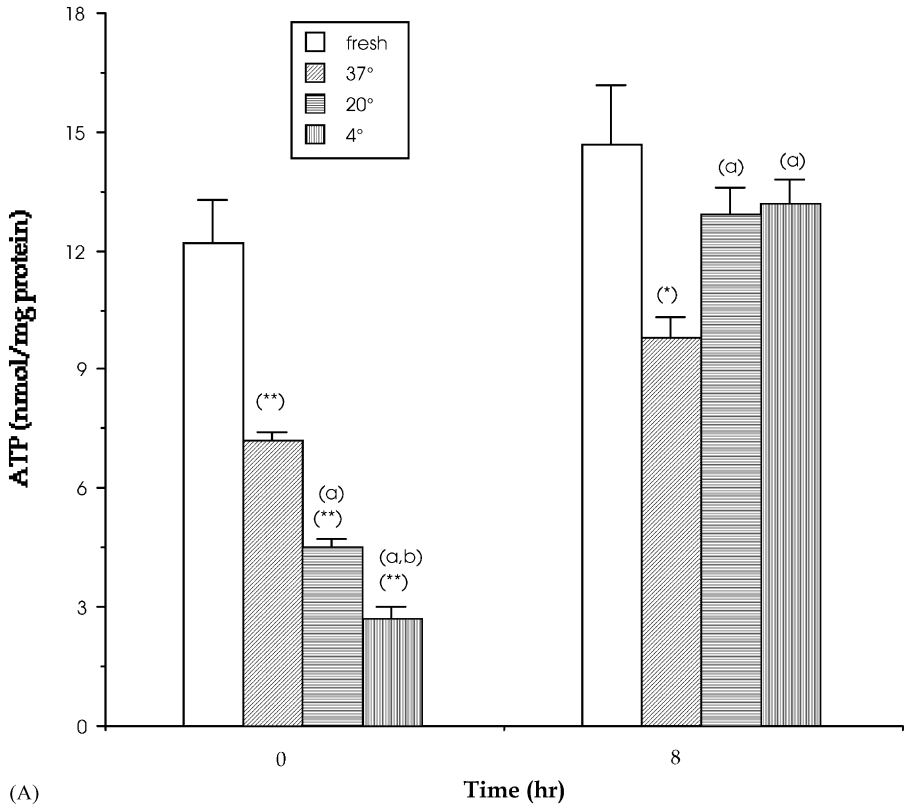


Fig. 4. The effect of Actinomycin D on CYP3A1, CYP3A2 and β-actin mRNAs at 20 and 37°. Liver slices were preincubated at 37° for 1 hr in the presence of actinomycin D (4 μM) and further incubated for 12 hr at either 20 or 37°. At the indicated times, RNA isolation and RT-PCR were performed as described under Section 2. The values were obtained by dividing O.D. units of CYP3A1/2 by O.D. units of β-actin.

midazolam, both metabolites were formed but they represented only 20% as rat liver microsomes. Interestingly, when CYP3A2 supersomes were used, 4-hydroxymidazolam was formed about 2.4 times than 1′-hydroxymidazolam, and such metabolites represented about 80–90% of that formed by rat liver microsomes. These results clearly showed that the formation of 4-hydroxymidazolam is tightly related to the activity of CYP3A2 protein.

4. Discussion

Liver metabolism is markedly influenced by temperature, a particular stress which play a critical role during liver transplantation. Indeed, we have previously shown that the temperature of reoxygenation, after overnight cold preservation, affected the survival of PCLS as well as its intermediary metabolism and phase-II conjugation reactions [24,25].



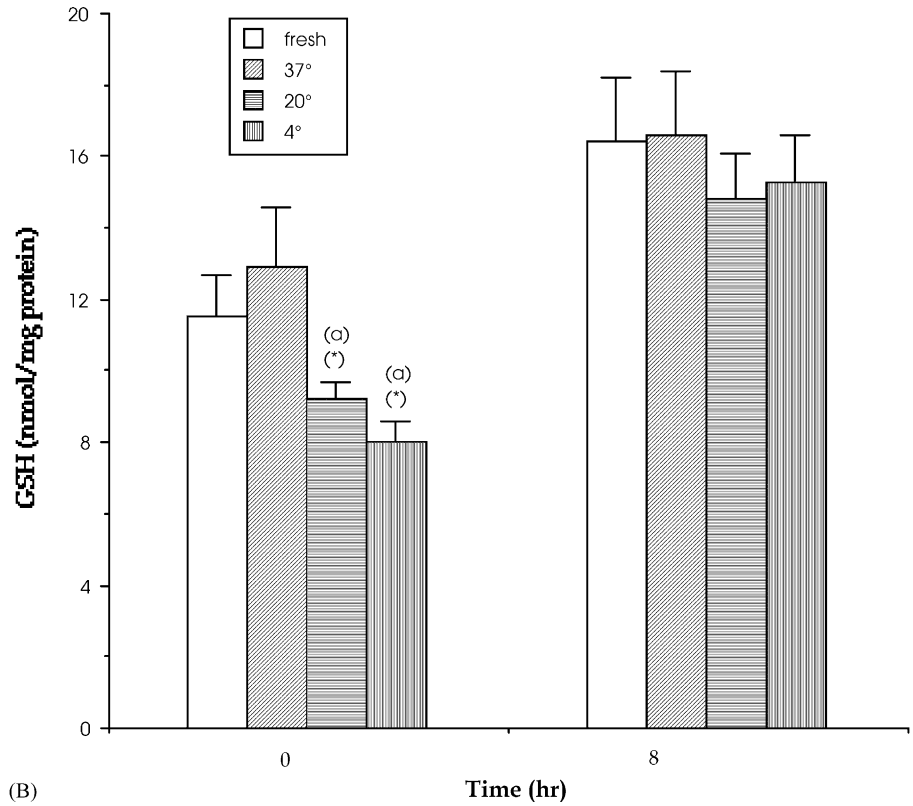


Fig. 5. The effect of incubation temperature on PCLS metabolic abilities. Liver slices were maintained for 20 hr at either 4, 20 or 37°. Afterwards, the slices were rinsed in saline and incubated for 8 hr at 37° in a fresh WME. At the beginning and at the end of 8-hr incubation, the ATP (A), GSH (B) and protein synthesis rates (C) were measured as described under Section 2. Experiments were performed in duplicate and values represent the mean ± SEM from at least three rats. The results were compared to freshly cut slices incubated for 8 hr at 37°. (*) $P < 0.05$ as compared to values in freshly cut slices; (**) $P < 0.05$ as compared to values in slices previously maintained at 37°; (***) $P < 0.05$ as compared to values in slices previously maintained at 20°.

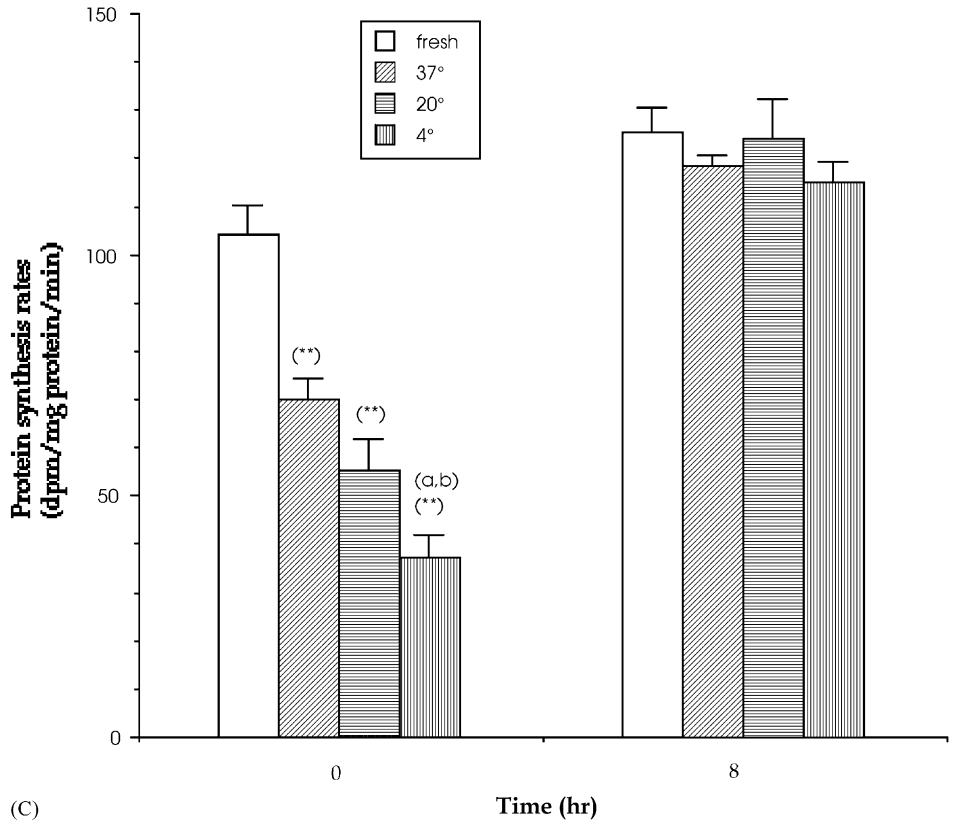


Fig. 5. (*Continued*).

Since temperature influences the oxidation of midazolam by CYP3A isoforms, both the CYP3A1 and CYP3A2 proteins and mRNA expression in liver slices were investigated after maintaining them for 20 hr at 4, 20 and 37°. It has long been known that CYP3A2 is a constitutive and a major CYP3A isoform [15,16], whereas the expression level of CYP3A1 is still a matter of controversy in untreated animals [11–14]. Since such discrepancies are related with the existence of allelic variants of CYP3A1, most of that clones have been named CYP3A23 [14]. Our results show that CYP3A1 protein was detected at the same level in all experimental groups and it was not affected either by the temperature or by the duration of the incubation. However, after 20 hr at 37°, the CYP3A1 mRNA was not longer detected showing no correlation between mRNA expression and the amount of CYP3A1 protein. Conversely, CYP3A2 protein and mRNA are affected by temperature. Indeed, neither protein nor mRNA transcripts were detected in PCLS maintained for 20 hr at 37°, while at 20° the protein was decreased by about 65% (as compared to freshly cut slices) but its mRNA was not affected. The amount of protein and mRNA in slices maintained for 20 hr at 4° was similar to that of freshly cut slices. These results suggest that temperature is playing a role in the stability of CYP3A2 protein and mRNA. Thus, the maintenance of liver slices at 4° results in protein and mRNA stabilisation while maintaining them at 37° leads to the loss of activity and a reduction of the levels of protein. Such a correlation, however, is not absolutely required since mRNA levels may not be associated with levels of protein/enzyme activity [15]. Furthermore, the results obtained after blocking transcription by actinomycin D indicate that a potential instability of mRNAs as a function of the temperature is unlikely involved in the differential loss of CYP3A2 protein at both 37 and 20°.

In agreement with previous reports [26–28], the results reported here show a time-dependent decrease of P450 activities. Interestingly, the metabolism of midazolam by either freshly cut slices or PCLS maintained for 20 hr at 4 or 20° results in a predominant formation of 4-hydroxymidazolam rather than 1′-hydroxymidazolam. The calculated 4-hydroxy/1′-hydroxy ratios were 1.9, 2.0 and 2.1, respectively. Such a regioselective activity was also observed in both rat liver microsomes [29], and rat cDNA-expressed systems [30], with a ratio between 4-hydroxy/1′-hydroxymidazolam of about 2.5–3.0. However, after 20 hr at 37°, PCLS retained only 20% of the initial CYP3A activity, and both metabolites (4- and 1′-hydroxy) were formed to the same extent with a ratio of 1.3, indicating that oxidation of midazolam by CYP3A was no more regioselective.

On the other hand, a well correlation was observed between loss of CYP3A2 protein and decreased midazolam oxidation by PCLS maintained for 20 hr at 37°. Furthermore, the CYP3A2 protein and the metabolism of midazolam, after 20 hr at 4°, were approximately the

same as those measured in freshly cut slices at zero time. Moreover, during the subsequent incubation for 8 hr at 37°, the amount of CYP3A2 protein of such previously cold preserved slices is gradually decreased, a finding which correlates well with a loss of midazolam oxidation in these slices. This predominant formation of 4-hydroxymidazolam as well as the changes in enzyme activity and amount of proteins raised the question, if the formation of 4-hydroxymidazolam is mainly catalysed by CYP3A2. Consistent with this hypothesis was the finding that both 4-hydroxy- and 1′-hydroxymidazolam were formed to a lesser extent by PCLS previously maintained at 37°, but such a metabolism was no longer regioselective and the amount of metabolites did not change with a subsequent incubation for 8 hr at 37°. Since no more CYP3A2 is available at such temperature, this residual metabolite formation must be dependent on CYP3A1, an isoform which is unaffected by temperature. Supporting that view, Perloff *et al.* [31], by using ketoconazole as CYP3A inhibitor, have shown that 1′-hydroxylation (but no 4-hydroxylation) of midazolam has a major CYP2C component in addition to CYP3A in both humans and mice. Moreover, our results obtained by using rat supersomes and those reported by Kobayashi *et al.* by using rat cDNA-expressed systems [30] strongly support the fact that 4-hydroxymidazolam is mainly metabolised by CYP3A2.

The P450 impairment at the level of mRNA, protein and enzyme activity is unlikely explained by loss of liver slices viability at 37° because at that temperature they retain their metabolic functions. Actually, in this present work, a complex and contradictory effect was observed in slices maintained for 20 hr at different temperatures. For instance, PCLS at 37° lost their capacity to oxidise midazolam but levels of ATP and GSH and protein synthesis rates were well preserved. Conversely, at 4 and 20°, PCLS retained their capacity to oxidise midazolam but they lost their metabolic ability, suggesting that cold preservation might cause some cytotoxicity. Supporting that view, it has been reported that cold hypoxia through different mechanisms, for instance, by activating protein kinases [32], by inducing an oxidative stress [33,34], may lead to cell death by apoptosis. Such a contradiction is, however, apparent because, under our experimental conditions, ATP and GSH levels and protein synthesis rates were restored, when cold preserved slices were further incubated at 37°, a temperature at which the cellular metabolism has been recovered.

A fascinating aspect of these results is the different sensitivity of CYP3A proteins regarding the incubation temperature. Since mRNAs of both CYP3A1 and CYP3A2 are equally affected by temperature and both proteins turn over with similar half-lives varying from 10 to 14 hr [35,36], differences in protein degradation rates at 37° might explain the loss of CYP3A2 protein. Actually, multiple proteolytic mechanisms for P450 degradation have been reported [37,38], and Korsmeyer *et al.* [39] reported that heme-modified P450 proteins need to be phosphorylated and ubiquitinated before being targeted to proteolytic disposal by the 26S proteasome. We hypothesise that CYP3A2 is modified first by such covalent reactions, and then degraded faster than CYP3A1. Actually, a similar mechanism has been proposed by Renwick *et al.* concerning the differential loss of CYP3A4 and 3A5 in cultured human liver slices [40]. This could explain why after 20 hr at 4° (due to enzymatic cold inactivation), liver slices retain the same ability as freshly cut slices to metabolise midazolam. Supporting that view, previous reports demonstrated that cold storage of hepatocytes had no effect on P-450-dependent activity [41,42]. Thus, by increasing the temperature, the activation of such proteolytic systems leads to a progressive loss of CYP3A2 protein. The molecular mechanism by which the incubation conditions might influence CYP3A protein stabilisation, deserves to be further explored.

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