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Kinetics of tienilic acid bioactivation and functional generation of drug–protein adducts in intact rat hepatocytes

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Abstract

Drug-induced autoimmune hepatitis is among the most severe hepatic idiosyncratic adverse drug reactions. Considered multifactorial, the disease combines immunological and metabolic aspects, the latter being to date much better known. As for many other model drugs, studies on tienilic acid (TA)-induced hepatitis have evidenced the existence of bioactivation during the hepatic oxidation of the drug, allowing the identification of the neoantigen of anti-LKM2 autoantibodies and the pathway responsible for its formation. However, most of these results are based on the use of microsomal fractions whose relevance to the liver in vivo still needs to be established. In the more complex intact cell environment, several endogenous processes may play a significant role on triggering the reaction and should therefore be considered. In this work we have characterised the kinetics of TA biotransformation in metabolically competent hepatocytes, the influence of TA bioactivation on physiological GSH levels, and the qualitative and quantitative profile of drug–protein conjugates generated in situ, as a function of exposure time. Results confirm that intact hepatocytes reproduce in vitro the metabolic sequence that leads to the functional generation of drug–protein adducts, in conditions that simulate clinical human exposure to TA. Metabolically competent cultured hepatocytes appear as a very promising approach to investigate the early preimmunological events of drug-induced autoimmune hepatitis, adequate to identify the conditions that may modulate the formation and specificity of drug–protein adducts in vivo, to study the hepatic disposition of the TA-protein targets, and to define the specific role of the hepatocyte in the origin of this adverse reaction.

Keywords: Primary cultured hepatocytes; CYP; Tienilic acid; Drug bioactivation; Glutathione; Drug–protein adducts

1. Introduction

Drug-induced autoimmune hepatitis is among the most severe idiosyncratic adverse drug reactions, and is characterised by an abnormal or inappropriate immune response directed specifically against the liver that follows the clinical exposure of susceptible individuals to certain drugs [1–3]. Considered multifactorial, the disease is the result of a complex multistep process that combines immunological and metabolic aspects [4–6], the latter being to date much better known. Our current understanding of the aetiopathogenesis of immune-mediated drug hepatotoxicity is largely based on two different but complementary working hypotheses, namely the ''hapten'' hypothesis [2,3,5,7] and the ''danger'' hypothesis [3,5,8]. Central to both hypotheses is the critical role of hepatic drug bioactivation as a key prerequisite for the initiation of drug-induced immune-mediated hepatotoxicity. Metabolic activation leads to the intracellular generation of chemically reactive intermediates able to act as haptens, but must also upregulate costimulatory signals—originated, e.g. from some type of cell stress or cell damage—in order to induce an effective autoimmune response. Despite the several clues provided in recent years, the reasons why so few individuals exposed to a drug that forms reactive metabolites actually develop the disease are not yet understood, and the exact nature and the mechanism(s) that upregulate ''danger'' in this context remain to be defined. The use of suitable in vitro experimental models,

Abbreviations: CYP, cytochrome P 450; L-NAME, N^G-nitro-L-arginine methyl ester; TA, 2,3-dichloro 4-(2-thienyl carbonyl) phenoxyacetic acid, tienilic acid; TBS, 50 mM Tris–HCl/150 mM KCl/1 mM EDTA buffer, pH 7.4

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able to reproduce the proposed sequential steps leading to drug-induced autoimmune hepatitis in man [2,4], will undoubtedly contribute to a better understanding of the complex molecular mechanisms underlying these undesirable adverse drug effects, which remain still unpredictable.

Tienilic acid (TA) is among the most extensively studied prototypic inducers of immune-mediated hepatotoxicity, in this case characterised by the presence of circulating anti-LKM2 autoantibodies [9]. Previous studies have clearly established that TA is mainly metabolised in the liver by human CYP2C9 and its rat orthologue (CYP2C11), with a similar and very high affinity, yielding to 5-hydroxy tienilic acid (5-OHTA) as the major stable end-product [10–12]. In the course of this hydroxylation, TA is metabolically activated to a highly electrophilic intermediate [13–15] which covalently binds to the CYP2C protein, but is also able to diffuse out of the CYP2C active site, then reacting with water, microsomal protein nucleophiles or GSH in the surrounding medium [12,14,16,17]. No significant contribution to TA metabolism has been demonstrated for human CYPs 1A, 2B or 3A [18]. Kinetic studies of the different members of the human CYP2C subfamily [19] have reasonably excluded a physiological role for CYP2C8, 2C18 and 2C19 under conditions of clinical human exposure to TA. In agreement with metabolic studies, immunoblotting studies have evidenced that serum containing anti-LKM2 antibodies exhibit a highly specific recognition for both, the native and the alkylated CYP2C11 [20,21] and CYP2C9 [18,22,23] isoforms, but do not immunoreact with rat or human CYPs belonging to the 1A, 2B, 2D, 3A or 4A subfamilies [20,24]. The relationship between the *CYP2C9* genotype and human susceptibility to TA-induced hepatotoxicity has not been yet specifically evaluated. However, as it has been the case for diclofenac [25] or cotrimoxazole [26], genetic polymorphism of the CYP2C9 enzyme is unlikely to represent an increased risk of TA hipersensitivity since the major allelic variants are associated with impaired metabolism, and thus reduced TA bioactivation, if compared to the wild-type [27]. From the studies cited above, it appears that in vivo exposure to TA should have necessarily lead to the alkylation of the CYP isoform responsible for hepatic TA biotransformation; CYP2C9 (CYP2C11 in the male rat liver) becomes thus the source and target of the TA bioactivated species in all the exposed subjects, but the TA-CYP2C adduct acts as a ''neoantigen'' only in those patients suffering TA-induced hepatitis [28].

As for many other model drugs (halothane, dihydralazine, phenytoin, diclofenac, etc.), studies with TA have been almost exclusively based on the use of microsomal fractions, isolated from rat and human livers or from heterologous systems expressing human liver CYPs, whose relevance to the liver in vivo still needs to be established. In the more complex intact cell environment, several endogenous processes involving different cellular compartments (i.e. CYP inhibition and/or induction, antioxidant defence mechanisms, protein target reactivity and accessibility, the cellular processing of the drug-modified proteins, or the concurrent induction of oxidative stress), may play a significant role on triggering the reaction and should therefore be also considered. The close analogies between CYP2C9- and CYP2C11-dependent TA bioactivation support the use of male rat liver parenchymal cells as an adequate cellular model of human TA biotransformation. We have recently developed a hepatocyte primary culture model that allows for an optimal preservation of CYP constitutive levels for prolonged times in culture [29,30]. This work was aimed to characterise: (i) the kinetics of TA biotransformation, (ii) the influence of TA bioactivation in hepatocyte GSH levels, and (iii) the quantitative and qualitative time–course profile of TA-protein alkylation upon prolonged exposure, in an intact cell environment. Results confirm that metabolically competent cultured hepatocytes reproduce the key features of hepatic TA oxidative metabolism, allowing the functional generation of the TA neoantigen in vitro, in conditions that simulate therapeutical human exposure to TA. Primary cultured hepatocytes therefore appear as a valuable tool for further mechanistic studies of the early preimmunological events in TA-induced hepatotoxicity.

2. Material and methods

2.1. Hepatocyte isolation and culture conditions

Hepatocytes from fed male Sprague–Dawley rats (180–250 g) were isolated by liver perfusion with collagenase (Boehringer Mannheim) and cultured on fibronectin-coated culture dishes (5.5 cm diameter, Nunc), as described previously [29,30]. Viability was assessed by the Trypan blue dye-exclusion test; only cell preparations with viabilities >90% were used in this study. Isolated hepatocytes were resuspended in Ham's F12 medium supplemented with 0.2% (w/v) BSA, 10^{-8} M insulin, 2% newborn calf serum and antibiotics (Gibco), and plated at a final density of 80,000 viable cells/cm^2. Unattached cells were removed by renewing the culture medium 1 h after plating. For an optimal preservation of initial CYP content, 1 mM N^G-nitro-L-arginine methyl ester (L-NAME, Sigma) was routinely added to the perfusion, washing and culture media, and kept present in the medium for the first 24 h of culture [29,30]. In some instances, hepatocytes were isolated and cultured in the absence of the nitric oxide synthase inhibitor; these are referred in the text as conventional or standard cultures. After the first 24 h of culture, monolayers were shifted to serum-free, L-NAME-free, insulin-supplemented Ham's F12 medium, containing or not 0.2% (w/v) BSA, and the experiments were initiated.

2.2. Cell homogenization and subfractionation

At the indicated times, isolated hepatocytes or cultured monolayers were gently washed with cold PBS, frozen

under liquid N_2 and kept at −20 °C until processed. Cells were scraped into ice-cooled 50 mM Tris–HCl/150 mM KCl/1 mM EDTA buffer, pH 7.4 (TBS), to provide 5–10 mg cell protein/ml. Whole cell lysates were obtained by brief sonication, followed by centrifugation at 1000 × *g* for 5 min at 4 °C to remove nuclei and unbroken cells. Microsomes were prepared by centrifugation (100,000 × *g*, 90 min, 4 °C) of the S9 fractions from crude homogenates. Microsomal pellets were resuspended in 50 mM Tris–HCl/20% glycerol buffer, pH 7.4, aliquoted and stored at −20 °C until used.

2.3. Tienilic acid 5-hydroxylation

Microsomal incubations were performed for a 20 min-period in conditions supporting maximal initial rates (1 μM P450, 0.2 μM TA and a NADPH-regenerating system), as previously described [18]. For assays in 24 h-cultured hepatocyte monolayers, TA was directly incorporated into the culture medium from 100× stock solutions made in the same medium and sterilized by filtration. The final TA concentration in the incubation was always verified by UV–vis spectrophotometry (ε_{306} = 14,000 M^{-1} cm^{-1}). 5-OHTA accumulation in the culture medium was quantified through UV–vis differential spectrophotomety [19,31] against the corresponding 0-time samples ($\varepsilon_{390-490}$ = 28,000 M^{-1} cm^{-1}).

2.4. Irreversible binding of TA to cellular proteins

All the experiments were performed in 24 h-cultured L-NAME-treated hepatocytes, in conditions providing maximal TA metabolic rates as defined by the kinetic studies (see Section 3). To quantify total covalent binding of TA metabolites to cellular protein, 300 μM [^{14}C] TA (sp. act. 57 μCi/μmol, a kind gift from F. Hoffmann-La Roche, Basel, Switzerland) was directly incorporated into BSA-free standard culture medium, and the incubation was prolonged for 24 h. At the indicated times, plates were quickly washed with ice-cooled PBS and the hepatocytes were scraped and homogenised in this same buffer through brief sonication, followed by centrifugation (1000 × *g*, 5 min, 4 °C) to remove nuclei and unbroken cells. Quantitative analysis of the irreversible binding of TA to whole cell protein was based on assays initially developed for microsomal incubations [17,18]. In some instances, after an 8 h-exposure to the drug, [^{14}C] TA was withdrawn from the culture medium, the monolayers were extensively washed with warm (37 °C) PBS then transferred to TA-free fresh Ham's F12 medium, and the culture was prolonged for an additional 16 h-period. The total amount of TA metabolised within a particular culture period was roughly estimated as the sum of nmol 5-OHTA produced plus the level of covalent binding (nmol TA-equivalents) achieved and, even in conditions of maximal metabolic rates, never exceeded a 10% of the total TA initially available in the culture medium.

2.5. Electrophoresis, Western blotting and autoradiography of TA-protein adducts

The quantitative amount and the pattern of cell proteins subject to covalent modification was analysed by SDS-PAGE, Western-blotting and autoradiography of the [^{14}C] TA-protein adducts formed as a function of time of exposure to saturating TA. 24 h L-NAME-treated cultures were exposed to 300 μM [^{14}C] TA in the conditions described above. At indicated times, monolayers were quickly washed in cold PBS and cells were harvested in 1 ml of TBS, pH 7.4, containing 1% SDS, and incubated for 5 min at 37 °C, then for 20 min at 4 °C, with gentle agitation. Whole cell lysates were clarified by centrifugation (16,000 × *g*, 15 min, 4 °C). Proteins were precipitated with ice-cooled acetone (80% v/v, final concentration), and kept overnight at −20 °C. Precipitated proteins were recovered by centrifugation at 16,000 × *g* for 15 min at 4 °C, dissolved in 0.2 ml of 50 mM Tris–HCl, pH 7.4, containing 1% SDS, and re-precipitated with acetone at −20 °C in the conditions described above. The washing steps were repeated in order to remove traces of entrapped or unbound [^{14}C] TA, until the radioactivity in the supernatants was equivalent to the background. The final protein pellets were carefully washed with 100% cold acetone, dried, re-dissolved in 0.1 ml of 50 mM Tris–HCl, pH 7.4, containing 1% SDS, and analysed for radiolabel and protein recovery.

Equal amounts of protein (15 μg/lane) were processed for SDS-PAGE (4–15% Ready gels, BioRad) under reducing conditions and transferred onto polyvinylidene difluoride membranes (PVDF, Amersham Biosciences) by semidry electroblotting (Trans-blot SD, BioRad). Coomasie blue staining of the PVDF membranes was performed to confirm a consistent protein loading between lanes, as well as to visualize the protein molecular weight markers. The membranes were air-dried, then exposed to autoradiographic films (BioMax MS, Kodak) with the aid of an intensifying screen (BioMax Transcreen LE system, Kodak) for 14–60 days at −70 °C, as required to obtain an optimal signal. The autoradiography and the Coomasie signal from the same membrane were digitalised and analysed using the Scion Image 1.63 (NIH) software. Band intensities of proteins of interest were found to be linear with respect to the exposure times used, with no additional bands appearing upon prolonged exposure. The data were corrected from the background and normalised to the total amount of protein/lane loaded. In some instances, the PVDF membranes used for [^{14}C] autoradiography were subsequently probed with an anti-CYP2C9 serum (see below) in order to determine the relative migration of the CYP2C11 protein.

2.6. Other assays

CYP holoenzyme was determined by carbon monoxide difference spectroscopy [32]. The relative content of

CYP2C11 apoprotein was determined by Western blotting, immunodetection and quantitative estimation against CYP2C9 standards in conditions providing linearity with respect to the amount of CYP protein applied, as previously described [30]. Rabbit polyclonal antibodies against human CYP2C9, able to cross-react with its corresponding rat orthologue, were kindly provided by Dr. P. Beaune (INSERM UMRS 490, Paris, France). Cellular GSH content was determined according to the fluorometric method of Hissin and Hilf [33], here adapted to micromethod (96-well plates). Cell integrity was checked by analysing hepatocyte viability (Trypan blue dye-exclusion test), aspartate aminotransferase leakage, and culture survival (cellular protein that remained attached to the plates), by standard procedures. The protein content was determined by the Lowry procedure [34].

2.7. Statistical analysis

Results are the mean ± S.D. for n independent cultures, as indicated. Statistical analysis was done by the Student's t test. A value of $P < 0.05$ was considered significant.

3. Results

3.1. Impact of hepatocyte isolation and culture conditions on TA 5-hydroxylase activity

In preliminary studies we have investigated the evolution of TA 5-hydroxylase activity in microsomal fractions from freshly isolated hepatocytes (0 time), and after different culture times in conventional conditions (no L-NAME treated). In microsomes from freshly isolated cells, 5-OHTA formation proceeded linearly for at least 1 h (Fig. 1A), with initial rates (0.37 ± 0.02 nmol 5-OHTA/min/nmol P450, $n = 6$) that were similar to those obtained in microsomal fractions from freeze-clamped male rat [11,31] or human [12,18] livers. However, when they are expressed per mg of microsomal protein, the ability to hydroxylate TA decreased sharply during the first 24 h in culture (Fig. 1B). The decline in TA 5-hydroxylation rates directly reflects the progressive loss of the constitutive CYP hemoprotein pool (Fig. 1B) that characterises hepatocytes in standard culture conditions [29,30], which is also associated with a selective and quick degradation of CYP2C11 apoprotein (Fig. 1C). The optimal preservation of functional CYP2C11 by 24 h of culture in L-NAME-treated hepatocytes (Fig. 2A) allowed for an accurate estimation of TA 5-hydroxylase activity in the intact cell on the basis of 5-OHTA accumulation in the culture medium (Fig. 2B). In the same conditions (not shown) TA 5-hydroxylation could not be detected in hepatocyte cultures or microsomal fractions prepared from female Sprague–Dawley rats, which express CYP2C12 instead of the major male rat liver isoform, CYP2C11.

3.2. Kinetics of TA 5-hydroxylation in metabolically competent 24 h cultured hepatocytes

The kinetics of TA 5-hydroxylase activity were determined following in vitro exposure of 24 h-cultured stable monolayers to varying TA concentrations. In both, conventional and L-NAME-treated cultures, 5-OHTA formation proceeded linearly for at least 8 h (Fig. 2B) and followed single Michaelis–Menten kinetics (Fig. 3A). At saturating substrate concentrations (500 μM TA), hydroxylation rates in metabolically competent hepatocytes

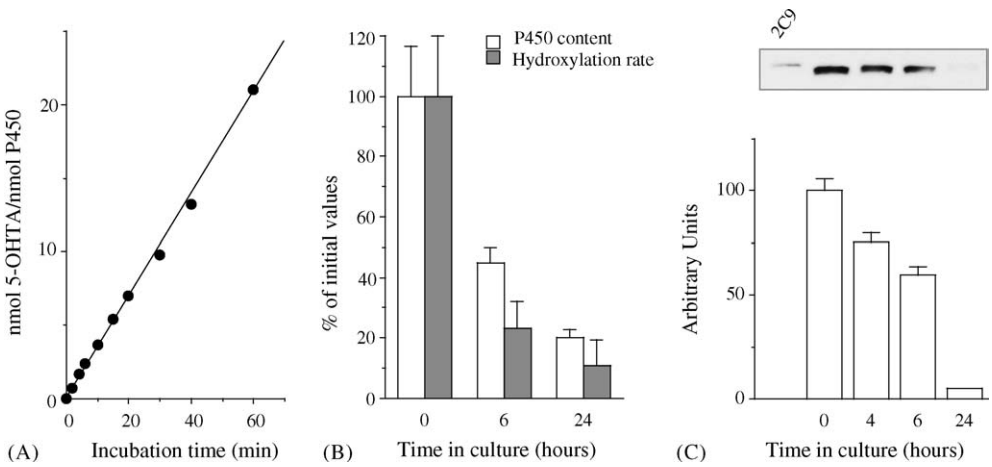

Fig. 1. CYP 2C11-dependent TA 5-hydroxylation in rat hepatocytes maintained in conventional culture conditions. Hepatocytes were isolated and cultured for 24 h in standard conditions (no L-NAME added). Microsomal fractions were analysed for 5-OHTA formation, CYP holoenzyme and CYP 2C11 apoprotein content. (A) Time–course of 5-OHTA production in microsomes from freshly isolated hepatocytes (representative assay). (B) Correlation of initial rates of TA 5-hydroxylase activity and CYP holoenzyme content, as a function of culture time. Data (mean ± S.D., $n = 3$) were referred to mg of microsomal protein, and are expressed as a percentage of their respective 0-time value (252.8 ± 50.8 pmol 5-OHTA/min/mg microsomal protein, and 0.68 ± 0.11 nmol P450/mg microsomal protein). (C) Immunodetection of CYP 2C11 apoprotein (5 μg protein/lane) in microsomes prepared after different culture times. Lower panel shows the densitometric analysis from three different cultures.

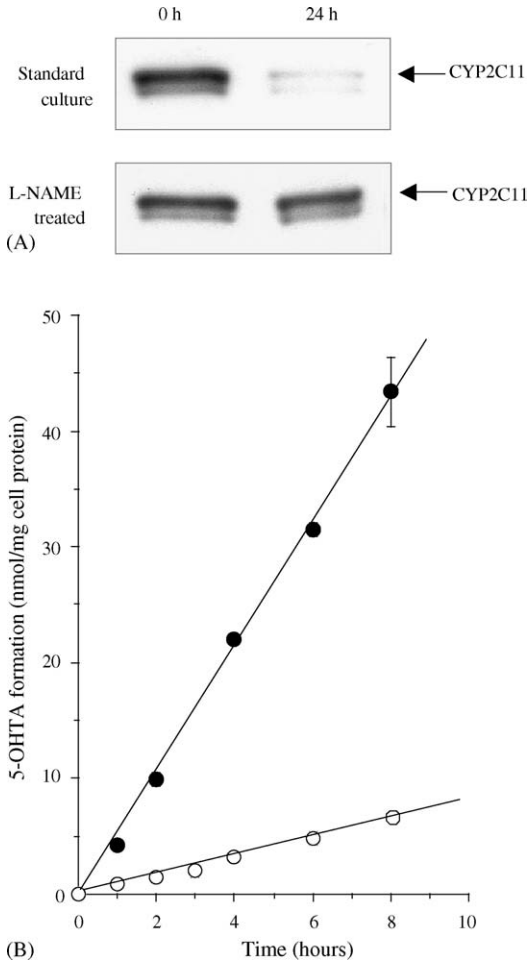

Fig. 2. CYP 2C11 apoprotein content (A) and TA 5-hydroxylase activity (B), in 24 h-cultured standard (○) vs. metabolically competent (●) hepatocyte monolayers. Isolation and culture conditions, as well as the analytical procedures, are described in Section 2. (A) Western-blot analysis of microsomal CYP 2C11 (2 μg protein/lane). A representative culture is shown. By 24 h of culture, CYP 2C11 represents a 5.0 ± 0.15% of initial in conventional cultures vs. a 92.5 ± 20% in L-NAME treated cultures ($n = 3$, see ref. [30]). (B) 5-OHTA accumulation in the culture medium as a function of time following exposure to 500 μM TA in the presence of BSA. Values are the mean ± S.D. from triplicate plates from one representative culture out of three.

represented a mean value of 92.5 ± 15.1 pmol 5-OHTA/min/mg cell protein ($n = 5$), five-fold higher than the rates exhibited by conventional 24 h-cultured hepatocyte monolayers (19.8 ± 5.9 pmol 5-OHTA/min/mg cell protein, $n = 5$). The lower rates in standard cultures reflect in fact a reduced functional CYP2C11 content, relative to that of L-NAME-treated cells. This was further confirmed by the 80% decrease (on average) in the estimated V_{max} values obtained in conventional versus L-NAME-treated monolayers (Table 1). However, the apparent K_m was equivalent in both culture models.

The apparent in vitro kinetic constants determined in the intact cell were clearly influenced by the presence of BSA in the medium (Fig. 3B and Table 1). The V_{max} estimates were analogous whether BSA was present or not, while the

Table 1
Apparent kinetic constants of TA 5-hydroxylation in primary cultured hepatocytes

	K_m (μM)	V_{max} (pmol/min/mg cell protein)	CL_{int} (μl/min/10^6 cells)
Conventional culture			
+BSA	83.9 ± 13.3	27.2 ± 1.4	0.40 ± 0.06
−BSA	26.6 ± 7.1*	25.2 ± 3.6	1.19 ± 0.31
L-NAME treated			
+BSA	84.2 ± 13.9	110.0 ± 10.1**	1.64 ± 0.26
−BSA	25.2 ± 4.4*	120.2 ± 11.3**	5.95 ± 1.01

The CL_{int} was derived from the V_{max}/K_m ratio. Values are the mean ± S.D. from three different cultures. Initial rates of 5-OHTA formation were determined (see Section 2) in 24 h-cultured monolayers incubated in the presence or absence of 0.2% (w/v) BSA.

* Significant difference in the K_m obtained ± BSA ($P < 0.001$).

** Significant difference in the V_{max} with respect to conventional cultures ($P < 0.001$).

apparent K_m was significantly increased (three-fold on average) if incubations were performed in the presence of BSA. On the basis of these results, the subsequent experiments were routinely performed in 24 h-cultured L-NAME treated cells, and in the absence of exogenous plasma proteins.

3.3. GSH depletion upon TA biotransformation

Twenty four hour cultured L-NAME treated hepatocytes exhibit GSH levels in the range of those determined in freshly isolated rat hepatocytes (11.5 ± 2.2 nmol/mg protein versus 11.9 ± 2.0 nmol/mg protein, respectively, $n = 6$) that remain stable for prolonged culture periods (not shown). Bioactivation of TA to a highly electrophilic intermediate was indirectly evidenced by a net decrease of the intracellular GSH pool. GSH depletion (with a maximal loss of up to 30%) was found to be time- (Fig. 4A) and dose-dependent (Fig. 4B), and actually correlated with TA hydroxylase activity for rates >40 pmol 5-OHTA/min/mg cell protein (Fig. 4C), that can be easily modulated in vitro through incubation against submaximal TA concentrations. Maximal GSH depletion rates (roughly estimated in ∼16 pmol GSH/min/mg cell protein) were observed within the first 0–2 h of exposure to saturating TA. No significant alterations on cell viability, membrane permeability, and monolayer survival were observed upon in vitro exposure to TA <500 μM (not shown); evidence of cellular damage was, however, significant upon prolonged exposure to higher TA concentrations (500–1000 μM).

3.4. Irreversible binding to hepatic proteins following in vitro bioactivation of TA

TA 5-hydroxylation was associated with the simultaneous time-dependent alkylation of intracellular proteins. The total binding to cell protein (Fig. 5A) increased sharply within the first 4 h of exposure to saturating TA, slowing

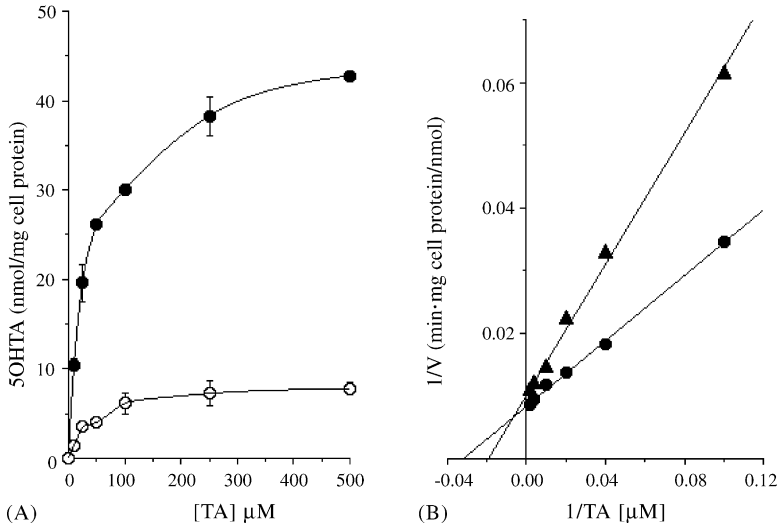

Fig. 3. Kinetics of TA 5-hydroxylation in 24 h-cultured hepatocyte monolayers. (A) Substrate concentration dependence curves in standard (○) and L-NAME-treated cultures (●). 5-OHTA was determined after a 6 h-incubation in BSA-free, hormone-supplemented Ham's F12 medium containing the indicated TA. Data are the mean ± S.D. of triplicate plates from one representative experiment out of three. (B) Lineweaver–Burk plots of TA 5-hydroxylation rates in L-NAME-treated cultures incubated in the presence (▲) and absence (●) of 0.2% (w/v) BSA. Data are means of duplicate plates from one representative culture.

down thereafter, to reach a maximum level within 8–24 h of exposure. From the cumulative curves, maximal rates of [^{14}C] TA incorporation to protein within the initial 0–4 h period were roughly estimated in 7.9 ± 1.3 pmol TA bound/min/mg cell protein, ~10-times lower than the TA 5-hydroxylation rates determined in parallel plates (81.3 ± 10.3 pmol 5-OHTA/min/mg cell protein). Fig. 5A suggests that the short initial period of linearity is followed by an apparent progressive decline in the rate of covalent binding over exposure time (6–24 h). As a result, the relative contribution of covalent binding to total TA metabolism represented ~22% within the first hour of exposure, but only a 4.5% by 4 h, and ~2.3% of total TA metabolized by 24 h of exposure (Fig. 5B). The decreasing covalent binding rates were neither due to a decrease in TA concentration in the medium, that remained saturating throughout the complete incubation period, nor due to a reduction in TA bioactivation rates, since TA oxidation to its end-stable product still proceeded linearly (Fig. 2B).

3.5. Characterisation of the TA protein-targets in the intact hepatocyte

Exposure of cultured hepatocytes to [^{14}C] TA in conditions supporting maximal TA biotransformation rates, correlated with the appearance of radioactivity irreversibly bound to intracellular proteins covering a wide range of molecular weights (Fig. 6A). The proteins preferentially labelled in whole cell lysates, exhibited apparent molecular masses in the range of 57–51 kDa (three bands), 48–44 kDa (two bands), 35–32 kDa (two bands) and 27 kDa (one band). The radiolabel associated with these proteins was already detectable after only 1 h of exposure to [^{14}C] TA, and increased significantly as a function of time (Figs. 6A and 7). Staining of the PVDF membranes (Fig. 6B) or polyacrylamide gels (not shown) with Coomasie blue, evidenced that the bands identified as major TA–protein adducts do not correspond to quantitatively important hepatic proteins, with the only exception of the 27- and 48-kDa polypeptides. Radiolabel was also associated to a lesser degree with other polypeptides (most notably the 155-, 130-, 95-, 72-, 62-, and 40 kDa) as well as with the low molecular weight protein front. However, the intensity of the [^{14}C] signal from these bands (relative to the background) was very low, which precluded further analysis. Although we cannot exclude the possibility that the minor bands reflect a specific covalent modification of the corresponding polypeptides, the lack of a time–course increase suggests that the signal corresponds to traces of entrapped [^{14}C] TA, still remaining in spite of the extensive washing performed.

Densitometryc analysis (Fig. 7) showed that the [^{14}C] signal associated to the major TA-protein targets increased rapidly as a function of time, to reach maximal steady-state levels between 8 and 24 h of incubation in the presence of TA. The time–course profile of the 48–44, 35–32, and 27 kDa bands resembles in fact the kinetics of quantitative [^{14}C] TA irreversible binding to total cell protein (Fig. 5A). TA binding to polypeptides in the 57–51 kDa range appears, however faster, achieving ~75% of the maximal (24 h) level by only 1 h of exposure to the drug. At this time, [^{14}C] TA incorporation to the 48–44, 35–32, and 27 kDa proteins was only ~40–50% of the maximal level achieved, suggesting that these adducts were formed more slowly. Interestingly, the molecular weight region involved (with two major bands at ~55 and 51 kDa) corresponds to the one expected for the CYP enzymes. Immunodetection of CYP2C11 in the PVDF membranes used for autoradio-

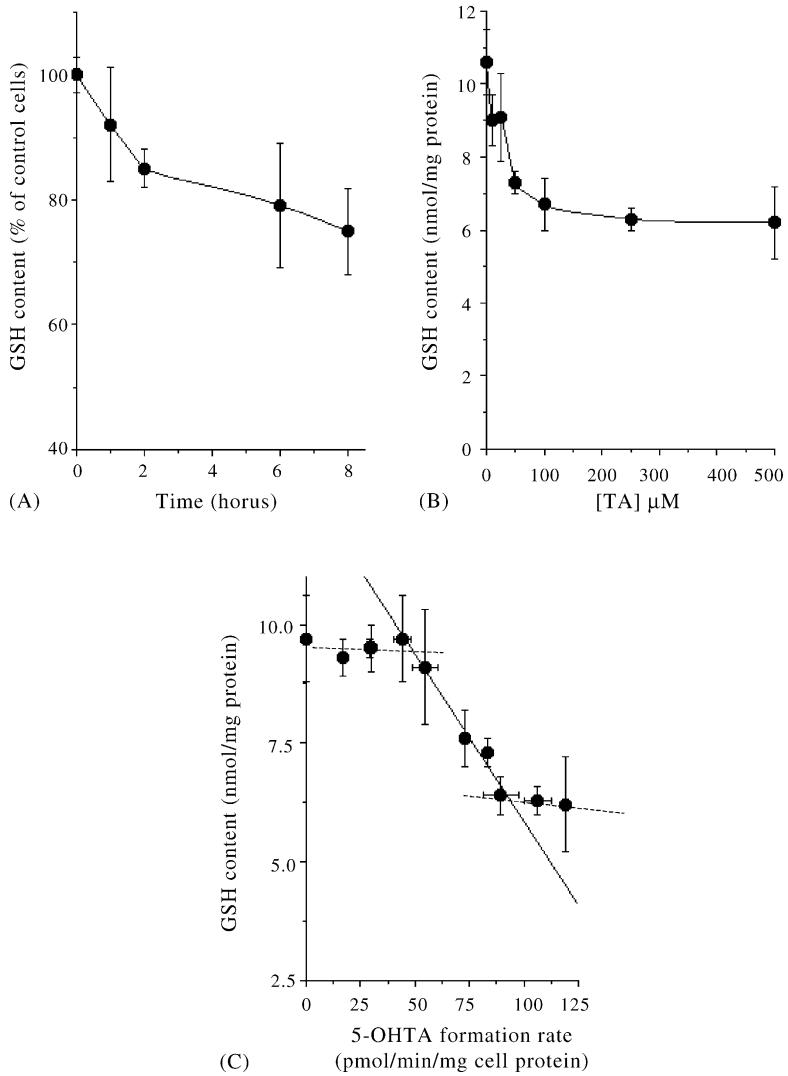

Fig. 4. GSH content after exposure to TA. 24 h-cultured, metabolically competent hepatocyte monolayers were shifted to L-NAME-free, BSA-free, insulin-supplemented Ham's F12 medium, containing the indicated TA concentrations. (A) Time–course evolution of intracellular GSH upon exposure to 500 μM TA. One representative experiment (mean ± S.D. from triplicate plates) is shown. Data are expressed as a percentage of their corresponding control values (an stable value of 12.0 ± 1.4 nmol GSH/mg cell protein, for the experiment depicted). (B) GSH content after 6 h of exposure to varying TA concentrations. One representative experiment (means ± S.D. from triplicate plates) is shown. (C) Correlation of GSH content with TA hydroxylation rates, both determined in the same plates. The figure compiles the data (mean ± S.D. from triplicate plates) obtained in three different cell cultures in which hepatocytes were exposed for 6 h to varying TA concentrations. Solid line shows the linear correlation (R value of 0.989) for rates between ~40 and 90 pmol 5-OHTA/min/mg cell protein.

graphy (Fig. 6B) confirmed that one of the major [^{14}C] TA-labelled bands comigrates in fact with CYP2C11.

3.6. Cellular processing of the TA–protein adducts[1]

To investigate the relative stability of TA–protein adducts in the living cells, 24 h-cultured monolayers were first exposed for 8 h to 300 μM [^{14}C] TA to allow the functional generation of TA–proteins in quantitative amounts, close to the maximal. At this time, TA was removed from the medium, and the culture was extended for an additional 16 h-period. As shown in Fig. 8A, the pre-formed pool of TA-modified cell protein decreased significantly upon withdrawal of [^{14}C] TA, with a mean percent reduction of ~33% at the end of incubation, while the covalent binding was still increased by an additional 20% if the substrate was kept present. Analysis of the labelled polypeptides in the region of 57–51, 48–44, 35–32, and 27 kDa (Fig. 8B), evidenced that all the major TA–protein adducts appear decreased in a similar extent. In the experimental conditions used, we have not been able to identify low molecular weight fragments (peptides) derived from these adducts. Taken together, results in Figs. 5A and 8 support the view that, once formed, the TA-adducts exhibit a rapid turnover, with half-lives expected to be less than 24 h.

[1] The term processing is used here to indicate any mechanism leading to a decrease in the intracellular protein-associated [^{14}C] TA (loss of the [^{14}C] TA label, protein degradation and turnover, or export of the modified proteins to the extracellular space).

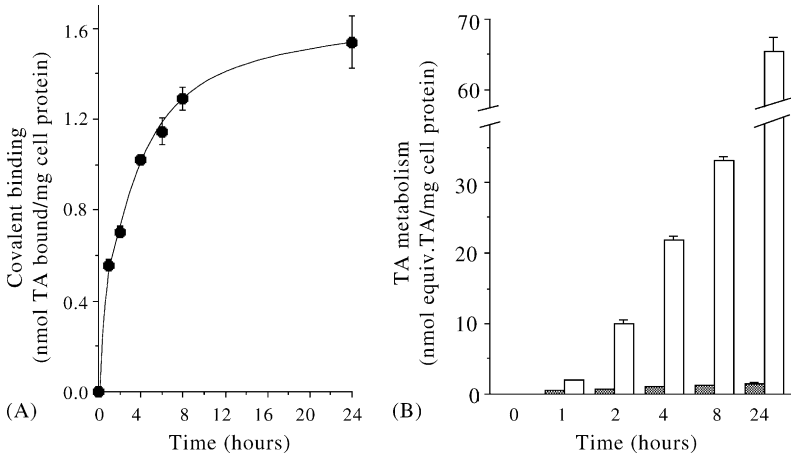

Fig. 5. Irreversible binding of TA to total cellular protein. 24 h-cultured L-NAME-treated hepatocytes were incubated in BSA-free, hormone supplemented Ham's F12 medium containing 300 μM [^{14}C] TA. Cell lysates were processed for quantitative analysis of the covalent binding, while 5-OHTA formation was analysed in the corresponding culture medium supernatants. Metabolic labelling was performed in duplicate plates from two different hepatocyte cultures. (A) Time–course of covalent binding of TA (mean ± S.D.). (B) 5-OHTA formation (clear bars) and TA covalent binding (shaded bars) from the same cultures (mean ± S.D.). By the end of the incubation period (24 h) TA concentration in the medium remained still saturating (∼256 μM).

4. Discussion

The use of microsomal fractions derived from different hepatic systems has undoubtedly facilitated the detection of drug bioactivation and protein alkylation for the typical model drugs known to induce autoimmune hepatitis. However, relatively little progress should be expected from the application of subcellular systems to further the understanding of the downstream consequences of these processes, i.e. the molecular events that occur in vivo following drug haptenisation of a protein, in which besides drug oxidation, several endogenous pathways and different cell compartments may be actively involved. At present, little is known about the cellular events that may influence the ''level'' of expression of drug–protein adducts in the liver, its nature or specificity, and their half-lives and turnover. In addition, the complex cellular pathways by which drug–protein conjugates finally lead to immune-mediated liver damage, and the determinant factors (genetic or environmental) of human predisposition, remain still unidentified.

Mechanistic studies aimed to investigate these questions at the molecular level can be supported by the use of properly validated in vitro cellular models. Undoubtedly, the first key for the suitability of an experimental system in this context is that it preserves the ability to bioactivate the drug in conditions relevant to the human liver in vivo, the second being its hepatic origin. The close analogies between CYP2C9 and CYP2C11 in TA bioactivation (see Section 1) support the use of male rat liver parenchymal cells

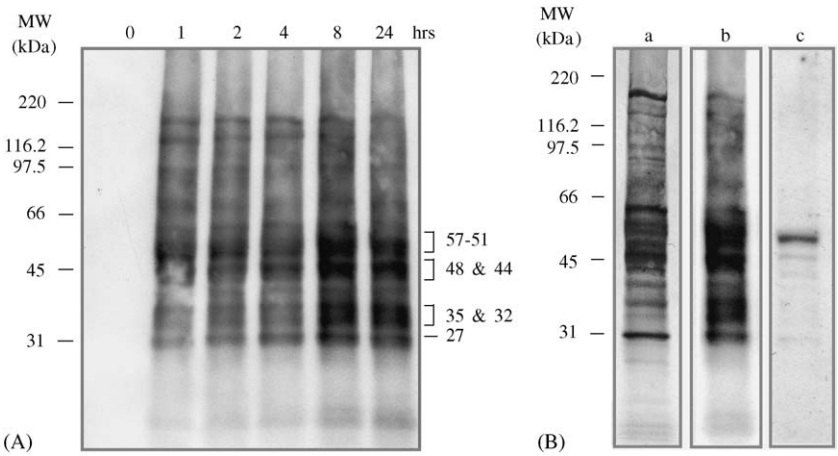

Fig. 6. Autoradiographic identification of hepatic TA–protein adducts. 24 h-cultured hepatocytes were exposed to 300 μM [^{14}C] TA in the conditions described in Fig. 5. Solubilised proteins from total cell lysates were processed and subjected to SDS-PAGE, Western-blotting and autoradiography, as described in the Experimental section. A representative culture is shown. (A) Time–course of protein-adduct formation (15 μg cell protein/lane, 8 weeks of exposure). The single and bracketed marks identify the apparent molecular masses of the major TA-adducts. (B) Coomasie blue staining (lane a), protein-associated [^{14}C] TA signal (lane b, 4 week-exposure), and immunodetection of CYP 2C11 protein (lane c) performed in the same PVDF membrane. Figure shows the specific pattern of the 8 h-incubation sample.

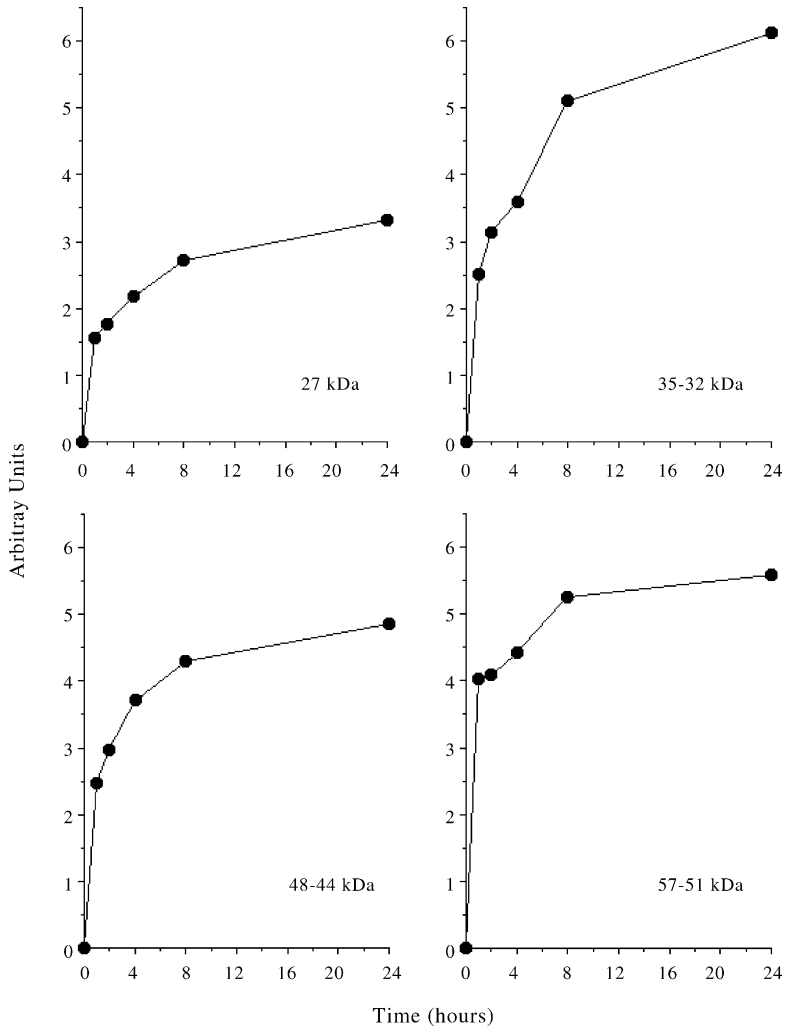

Fig. 7. Densitometric quantification of the major TA-protein adducts. Data corresponds to samples in Fig. 6. The time–course profile was equivalent whatever the exposure period used. Because the specific TA-protein bands located on the 35–32, 48–44 and 57–51 kDa range were not sharply resolved on the autoradiographic film (Fig. 6A), the peak areas corresponding to these proteins were determined as an average.

to mimic human liver TA biotransformation. Freshly isolated hepatocytes, however, do require ∼24 h in primary culture to fully restore the normal hepatic morphology and differentiated functions; this "recovery" period results essential for the studies to be carried out, since it allows the hepatocytes to reverse the very important phenotypic changes induced by the isolation procedure itself, i.e. metabolic damage, strong oxidative stress and activation of the MAP kinase and NF-κB signalling pathways [29,35,36], that otherwise would significantly interfere with the formation and/or the intracellular processing of drug–protein conjugates. But at the same time, the early culture period is associated with the rapid and irreversible loss of CYP hemoproteins (see, e.g. Fig. 1B), a well-known limitation of hepatocyte cultures in drug metabolism studies. The very early quantitative loss of CYP2C11 in culture [30] is particularly critical for TA biotransformation studies (Fig. 2) since it prevents the in vitro generation of the TA bioactivated species in quantitative amounts, thus precluding a sensitive detection of TA–protein adducts in 24 h-cultured stable monolayers. Recently, we have shown that attenuation of nitrosative stress during the isolation and the very early culture hours provides the simplest means to efficiently prevent CYP degradation so that, after 24 h of culture, L-NAME treated hepatocytes do preserve the initial constitutive CYP levels [29] and the CYP profile of the rat liver in vivo [30]. The present study provides the first detailed data on the main features of in vitro TA biotransformation in metabolically competent hepatocytes under controlled, highly reproducible conditions. As intact cells are used, manual handling and enzyme damage are minimised and cell organelles are largely preserved, so that the concurrent metabolic pathways can be operative in their native environment, in conditions relevant to the hepatocyte in vivo.

Kinetic studies confirmed that TA hydroxylation rates: (i) correlate directly with the level of expression of functional CYP 2C11 (Figs. 1 and 2), with no apparent limitations imposed by TA uptake, enzyme accessibility or endogenously supplied oxygen or cofactor(s), (ii) depend on the TA concentration present in the medium (Fig. 3),

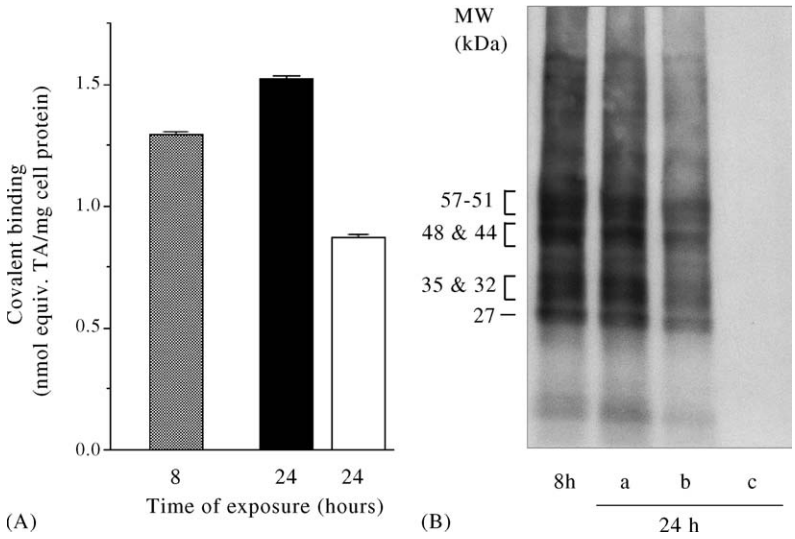

Fig. 8. Turnover of TA-protein adducts in the intact hepatocyte. 24 h-cultured hepatocyte monolayers were exposed for 8 h to 300 μM [^{14}C] TA; after that time, some plates were collected (8 h values) while the remaining culture plates were incubated for a further 16 h-period (24 h values) in the presence or absence of [^{14}C] TA. Whole cell lysates were processed for (A) the quantitative analysis of total irreversible binding to cell protein, and (B) the autoradiographic detection of the major TA-protein adducts (15 μg cell protein/lane, 4 weeks of exposure). Shaded bar, 8 h: protein bound TA after 8 h of exposure; Dark bar, lane a: hepatocytes exposed for the whole period (24 h) to 300 mM [^{14}C] TA; Clear bar, lane b: hepatocytes pre-exposed for 8 h to [^{14}C] TA, then cultured for 16 h in the absence of the substrate; Negative controls, lane c: hepatocytes cultured for 24 h in the absence of TA. Values (mean ± S.D.) are derived from triplicate plates from a single experiment.

and (iii) are saturated at clinically relevant TA concentrations, i.e. close to the maximal plasma levels reached in man (75–300 μM TA, depending on the dosage) 2 h after the administration of a single oral dose [37]. Interestingly, pharmacokinetic studies [37] have suggested that the hepatic clearance of TA in man might be saturated at the highest therapeutic dose (1000 mg). Comparison of the apparent kinetic constants (Table 1) shows that in metabolically competent hepatocytes, the V_{max} for TA 5-hydroxylation is five-fold higher than in conventional cultures whereas the K_m is equivalent; this justifies the higher CL_{int} values found in L-NAME treated cells, according to which TA might be considered as a high-turnover drug [38]. In both, conventional and metabolically competent cultures, the apparent K_m results significantly increased if the incubations are performed in the presence of BSA while the V_{max} remains unchanged; the observed increase is a likely consequence of the extensive binding (>98%) of TA to plasma protein [37] lowering its effective concentration in the medium. Finally, a decrease of the apparent affinity for TA in the intact cell relative to that shown by microsomal fractions from rat (14 ± 2 μM, ref. [31]) and human (10–15 μM, ref. [12]) livers, suggests a drug uptake limitation, including the possibility of transporter involvement.

The proportion of the chemical converted into a reactive species (versus end-stable product) and the counteracting action of endogenous detoxifying mechanism(s), may both play a critical role as metabolic determinants of the yield of drug–protein conjugates formed in the intact cell. The bioactivated TA species is produced as an intermediary in the own sequence leading to CYP2C-dependent 5-hydroxylation [16,18]. Thus, rates of TA bioactivation in cultured hepatocytes can be reasonably assumed to be at least equivalent to the measured 5-hydroxylation rates, and to proceed linearly for the whole duration of exposure. Results have evidenced that TA hydroxylation is paralleled by the functional generation of TA–protein conjugates (Fig. 5) and the concurrent time-dependent decrease on hepatocyte GSH (Fig. 4). Net GSH depletion upon in vitro exposure to TA actually correlates with TA 5-hydroxylase activity (i.e. TA bioactivation rates) for rates >40 pmol 5-OHTA/min/mg cell protein, and is maximal against saturating TA, i.e. hydroxylation rates equivalent to the apparent V_{max}. GSH conjugation by either, enzymatic or direct reactions, necessarily involves the irreversible loss of GSH molecules that can only be replaced via de novo synthesis. Primary culture represents a dynamic system in which hepatocytes, kept in the presence of the adequate aminoacid precursors provided by the culture medium, do preserve the ability to resynthesise GSH upon oxidative injury, and do maintain high stable GSH levels (equivalent to ~3–4 mM GSH). In our hands, GSH de novo synthesis rate (not shown) is in the range of 19.3 ± 0.9 pmol GSH/min/mg cell protein (n = 3). Taken together, these results: (i) support the view that, in physiologically relevant conditions for TA bioactivation, net GSH loss is only significant when GSH synthesis rate is overcome, (ii) confirm the reactivity of the TA activated species towards GSH in the native cell microenvironment, first suggested from studies with microsomal fractions [11,12,16,17], and (iii) indirectly suggest that conjugation with GSH would pick up a significant fraction of the reactive species. No specific studies have been carried out to confirm the

formation of stable TA–GSH conjugates, but some evidence in the literature supports the view that the conjugation pathway may play a significant role on hepatic disposition of TA in vivo. The earliest pharmacokinetic studies in man and rat [37,39] already established that a minor but significant fraction of the administered dose was excreted in the bile as (by then) unidentified conjugated products, the agents involved being presumably aminoacids (GSH or cysteine). Recent studies [15] have in fact identified the chemical structure of two major TA–GSH adducts formed during the metabolic activation of TA by reconstituted recombinant CYP2C9 and rat liver microsomes. Collectively, the aforementioned results suggest that, even at low TA dosage regimens, any impairment or imbalance on hepatic GSH biosynthesis or GSH content (caused, e.g. by oxidative stress, inflammation, or concomitant drug therapies) might contribute to a decreased conjugation and, therefore, to increased levels of the reactive metabolite available to freely interact with intracellular macromolecules; both, the TA activated species or GSH depletion itself, could in turn cause sufficient cell stress to result in a danger signal [3,8] without still causing serious direct toxicity. This question is now under investigation in our laboratory.

The time–course of quantitative TA irreversible binding to cell protein clearly indicates that, contrary to what might be expected, TA bioactivation does not need to exhaust the hepatocyte GSH pool to efficiently react with its intracellular protein targets (Fig. 4A and 5). This can be reasonably explained by either, the higher reactivity of the activated species towards the specific proteins involved, or the inability of GSH to gain access to these proteins or the subcellular compartment involved. Characterisation of the hepatic proteins subject to covalent modification was here based on radiochemical labelling. This approach, unlike immunodetection, excludes the risk that some adducts may go undetected as a result of unique differences in antigenicity, and it facilitates the direct comparison of the signal intensity among different proteins as well as their relative increase as a function of time. Several TA-adducts that were expressed in a time-dependent manner (bands in the range of 35–32 kDa $\geq$ 57–51 kDa $>$ 48–44 kDa $>$ 27 kDa, in order of signal intensity) were identified as major targets, together with some other minor bands distributed throughout the molecular weight range (Fig. 6). It is noteworthy that the pattern of TA–protein adducts expressed in the intact hepatocyte differs markedly from the pattern defined in previous studies [17,21,22] where a selective, almost exclusive, modification of the CYP2C band was initially established, consistent with the CYP catalyst being the antigen recognized by anti-LKM2 antibodies. However, it should be remarked that in the former studies, mainly based on immunochemical detection of protein-bound TA, only the microsomal fractions were routinely investigated. To our knowledge, this work provides the first experimental evidence that in the more complex intact cell environment, CYP2C is not the sole target of TA since many different proteins, likely to involve different cell compartments, are capable of reacting at comparable degrees with the bioactivated TA species, even if a physiological GSH level is present.

Kinetics of TA binding to proteins in the 57–51 kDa range show that these adducts, likely to involve CYP2C11 according to comigration studies, are quantitatively generated at very early exposure times, while the protein conjugates of 35–32, 48–44, and 27 kDa occur later in the time–course. Preferential adduction of the CYP involved in TA bioactivation confirms that, in the intracellular microenvironment, the reactive metabolite near the CYP2C11 active site is less efficiently scavenged by cytoprotective antioxidants such as GSH (mainly present in the cytosol), as already evidenced in microsomal fractions [16,17]. Nevertheless, results suggest that a significant proportion of bioactivated TA is also able to migrate from CYP 2C11, and to react sequentially with neighbouring proteins in the endoplasmic reticulum (possibly other CYPs), with proteins located in other cell compartments, as well as with GSH itself. Current research in our laboratory is directed to characterise the subcellular distribution and the identity of the major hepatic proteins targeted, as a necessary step to further understand the potential role of their human orthologues in TA-induced hepatitis.

Finally, the time–course profile of protein alkylation in conditions in which TA 5-hydroxylation was proved to proceed at constant rates, shows that the amount of [^{14}C] TA incorporated increases rapidly as a function of time to reach maximal steady-state levels by about 8–24 h of continuous exposure (Fig. 5), a pattern that affects similarly the eight polypeptides identified as major TA–protein adducts (Fig. 7). These data strongly suggest a limited availability of the TA specific targets (i.e. access or saturability of the aminoacid residues involved), and/or a rapid turnover of the alkylated proteins in the intact cell context. In support of this view, preliminary results in this work (Fig. 8) indicate that once formed, the major TA-adducts are not persistent within the hepatocyte which therefore seems able to process[1] efficiently the chemically modified proteins which, in addition, appear short-lived. A similar time–course has been shown in rats in vivo for the formation and disposition of the hepatic microsomal TA-CYP2C11 adduct [21], where the maximal levels were achieved 2 h after the administration of a single i.p. TA dose (the earliest time investigated), reached a plateau from 2 to 6 h, and then exhibited a progressive decline with an apparent $t_{1/2}$ of about 12 h.

The current available animal models of drug-induced immunoallergic hepatitis are aimed to reproduce the clinical syndrome as it occurs in humans, and may represent an invaluable tool to characterise the nature of the immunological events underlying the autoimmune response [40–42]. On the other hand, metabolic processes relevant to formation and disposition of drug–protein conjugates in

the liver may be easily investigated in vitro, provided that complete living cells are used. Results in this study support the suitability of metabolically competent (i.e. CYP-expressing) cultured hepatocytes in modelling in vitro the metabolic sequence that leads to the functional generation of hepatic TA–protein adducts in vivo, in conditions that reasonably simulate therapeutical human exposure to TA. As shown, TA irreversible binding to hepatic proteins cannot be avoided by a physiological GSH level, and appears rather selective, if compared, e.g. to drugs such as paracetamol [43] or the TA isomer [17], with CYP2C11 representing a primary and very early target for alkylation and a very limited number of individual proteins additionally targeted at similar levels. As the complex in vivo environment is well preserved, enabling a dynamic balance between TA bioactivation, detoxication of the reactive species and covalent binding to intracellular protein targets, primary cultured hepatocytes appear as a valuable in vitro tool to investigate: (i) the identity and function of the target proteins, (ii) the circumstances that may modulate the amount and the specificity of drug–protein conjugates in the liver in vivo, (iii) their potential to cause cell stress or creating a danger signal, as well as (iv) any biological consequence of this binding to cell homeostasis. To date, the few formal studies dealing with hapten-specific processing (see, e.g. halothane [44], diclofenac [45,46] or TA [21]) have routinely analysed hepatocytes freshly isolated from rats exposed in vivo to the model drugs. As mentioned above, in these conditions the normal cell processing of drug–protein conjugates or protein turnover may result significantly interfered due to the pro-inflammatory response induced by the collagenase-based isolation procedure [29,35,36]. Metabolically stable 24 h-cultured hepatocytes appear therefore as a unique physiological model, more realistic than microsomal fractions, for the detailed investigation of the ultimate intrahepatic fate of TA–protein adducts, in studies aimed to define the specific role of the hepatocyte in the origin of the autoimmune adverse reaction.

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