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Toxic effects of tacrine on primary hepatocytes and liver epithelial cells in culture

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Abstract

Administration of tacrine (THA) for the treatment of Alzheimer's disease results in a reversible hepatotoxicity in 30–50% of patients, as indicated by an increase in transaminase levels. However, the intracellular mechanisms underlying such a toxicity have not yet been elucidated. In this study, we performed short-term and long-term *in vitro* treatments on primary human and rat hepatocyte cultures as well as on nonparenchymal rat liver epithelial cells (RLEC), known as CYP1A-deficient cells. Cell ultrastructure was analyzed under different conditions and the release of lactate dehydrogenase (LDH) was used to evaluate cytotoxicity. The effects of THA on protein synthesis, intermediary metabolism and reduced glutathione (GSH) level were also determined in rat hepatocytes. THA induced dose-dependent toxic effects in liver parenchymal and nonparenchymal cells, with human hepatocytes being less sensitive. This toxicity appeared to be unrelated to metabolism of THA since similar effects were observed in rat hepatocytes and RLEC, in which THA metabolism was found negligible. Ribosome aggregation appeared only at high concentrations (>1 mmol/L) and was not specific to hepatocytes. Therefore, the THA-induced decrease in protein synthesis observed at lower concentrations was likely not related to this alteration. ATP and glycogen levels as well as GSH content were reduced upon THA. However, while glycogen level decreased at THA doses similar to those inducing an increase in LDH release, the fall in ATP and GSH contents occurred at higher doses. Thus, glycogen level in hepatocytes appeared to be a more sensitive indicator of THA toxicity than were ATP and GSH levels. We also found that protein synthesis started to decrease at THA doses that were still ineffective on LDH release. This might suggest that the decrease in synthesis of one or several proteins upon THA treatment represents the early signal leading cells to death.

Abbreviations: CYP, cytochrome P450; FCS, fetal calf serum; GSH, reduced glutathione; GST, glutathione-*S*-transferase; LDH, lactate dehydrogenase; RLEC, rat liver epithelial cells; THA, tacrine

Introduction

Tacrine (1,2,3,4-tetrahydro-9-aminoacridine; THA) is a centrally active acetylcholinesterase inhibitor currently administered to thousands of patients for the treatment of Alzheimer's disease (AD) (Farlow et al., 1992; Knapp et al., 1994). Unfortunately, tacrine has been reported to induce reversible increases in serum transaminase activity suggestive of hepatic injury in 30–50% of the patients (Forsyth et al., 1989; O'Brien et al., 1991; Watkins et al., 1994). However, the mechanisms underlying the hepatotoxicity of tacrine remain to be elucidated. Tacrine has been demonstrated to be metabolized by CYP1A2 and the main stable metabolites formed by CYP1A2 are 1-, 2-, 4- and 7-hydroxytacrine (Madden et al., 1993; Spaldin et al., 1994). Among the hypotheses put forward to explain the toxicity of tacrine, a role for metabolites produced by CYP1A enzymes has been suggested. With respect to this, it has been shown that human liver and 3-methylcholanthrene-treated rat microsomes produce more covalently bound tacrine than control rat microsomes (Madden et al., 1993; Bezek et al., 1996). However, general observations do not support the hypothesis that tacrine hepatotoxicity is related to its activation. First, tacrine has been found to be equally cytotoxic to rat hepatocytes and to HepG2 human hepatoma cells, known to lack CYP1A2 activity, and its toxicity is not prevented by the presence of CYP1A2 inhibitors (Viau et al., 1993; Berson et al., 1996). Second, tacrine toxicity remains unaffected in rat isolated hepatocytes after GSH depletion by diethylmaleate treatment, a maneuver known to inhibit GST activity; this points to the noninvolvement of electrophile formation in THA toxic effects (Dogterom et al., 1988). Third, GST μ1-deficient and GST μ1-positive individuals are equally susceptible to tacrine (Green et al ., 1995).

Another hypothesis, raised by Fariss and coworkers (1994), involves effects of tacrine on ribosomal function and protein synthesis. However, it is noteworthy that these effects were observed following short-term treatments with high doses of tacrine, thus making questionable the possible relevance of such effects to what really occurs in treated patients. Recently, Berson et al. (1996) have shown that tacrine affects mitochondrion function in rat hepatocytes and human lymphocytes. In this latter study, the effects were observed upon treatment with clinically relevant doses of tacrine, thus pointing to a possible involvement of mitochondrion dysfunction in tacrine-induced liver disease.

The present study was carried out to further analyze the intracellular mechanisms underlying the hepatotoxicity of tacrine. To this end, we performed short-term and long-term *in vitro* treatments on primary human and rat hepatocyte cultures as well as on nonparenchymal rat liver epithelial cells shown to be undifferentiated CYP1A-deficient cells. Cell ultrastructure was analyzed under different conditions of treatment, and the lactate dehydrogenase (LDH) release was used as a marker of tacrine toxicity. The effects of tacrine on protein synthesis, intermediary metabolism and reduced glutathione (GSH) level were also determined in rat hepatocytes.

Materials and methods

Reagents

Williams' E medium was from Eurobio (France). Fetal calf serum was from Dominique Dutscher SA (Brumath, France). Collagenase was from Boehringer Mannheim (Germany). Tacrine, dimethyl sulfoxide (DMSO), and glucose (Trinder) kit (to determine glycogen concentration) were purchased from Sigma (St Louis, MO, USA). LD kit (for LDH determination) was from Bayer (France).

Lumit reagents (to measure intracellular ATP) were from Lumac B.V. (The Netherlands). Bioxytech GSH-400 kit (to measure reduced glutathione) was from Oxis International (USA). [^{14}C]Leucine (specific activity 342 mCi/mmol) was from Amersham (UK).

Cell isolation and culture

Hepatocytes were isolated by a two-step collagenase perfusion procedure as described previously (Guguen-Guillouzo et al., 1983). Human hepatocytes were obtained from normal liver fragments resected from primary and secondary tumors. In the present study, two human livers (HL1 and HL2) were used for both cytotoxicity assays and electron-microscopic observations. All experimental procedures were approved by the National Ethics Committee. Rat hepatocytes were obtained from the liver of male Sprague-Dawley animals weighing around 200 g.

Following dissociation, cell viability was estimated by the trypan blue exclusion test and was in the range 75–85% for human hepatocytes and above 80% for rat hepatocytes. Unless stated otherwise, liver parenchymal cells were routinely seeded at densities of 15×10^4 cells/well in 24-well microplates (LDH determination) and 3×10^4 cells/well in 96-well microplates (glycogen and ATP determinations) in Williams' E medium supplemented with 10% fetal calf serum (FCS) and (per ml) 5 IU penicillin, 5 μg streptomycin, 1 mg bovine serum albumin and 500 μg bovine insulin. The cells were cultured at 37°C in an atmosphere of 5% CO_2 and 95% air. The media for rat and human hepatocytes were renewed after 4 h and about 24 h, respectively, to remove unattached cells. The fresh medium was then supplemented with 5 μmol/L hydrocortisone hemisuccinate. Treatment with tacrine was generally started at that time and, when long-term treatments were performed, the medium was renewed every day thereafter.

Rat liver epithelial cells (RLEC) were obtained by trypsinization of the livers of 10-day-old Sprague-Dawley rats, as described elsewhere (Morel-Chany et al., 1978) and were used between passages 10 and 25. They were cultured in Williams' E medium supplemented with 10% FCS. RLEC were treated with tacrine after reaching confluency.

Electron microscopy

Cell cultures were washed with chilled phosphate-buffered saline (PBS), fixed in 2.5% glutaraldehyde in 0.1 mol/L cacodylate buffer (pH 7.4) for 4 min at 4°C. Finally, they were postfixed in 1% osmium tetroxide solution. Cells were then dehydrated in graded ethanol and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and examined with an electron microscope.

Cytotoxicity assays

Tacrine-mediated cell death was determined by measuring the leakage of cellular lactate dehydrogenase (LDH) into the medium. LDH activity was measured both in media and in cell extracts using a kit for LDH determination (LD kit, Bayer). Before addition of the reagents, cell monolayers were lysed in 0.2% Triton X100 in water for 15 min at room temperature. Optical density was then measured at 340 nm. To determine the dose of THA inducing 50% cell mortality (IC_{50}), the basal extracellular LDH/total LDH ratio (LDH_e/LDH_{tot}; i.e., in absence of THA) was assigned to zero mortality, while ratios above 0.9 were assigned to 100% mortality (confirmed by light-microscopic observations).

Metabolism studies

Culture media of primary rat hepatocytes (10^6 cells per 35 mm diameter Petri dish) and of RLEC treated with 0.1 mmol/L THA for

24, 48 or 72 h were collected and stored frozen (–80°C) for later high-performance liquid chromatography determinations of THA and different metabolites (1-, 2-, 4-hydroxytacrine) to further characterize the metabolic competence of these cells.

Protein synthesis

Protein synthesis was measured by incorporation of [^{14}C]leucine. For this assay, hepatocytes were seeded at a density of 10^6 cells per 35 mm diameter Petri dish. Four hours before harvesting the cells, the monolayers were washed with PBS and incubated in a serum-free, leucine-free medium supplemented with 0.2 μCi/ml [^{14}C]leucine. At the end of the incubation time, media and cells were collected separately, precipitated with trichloroacetic acid (TCA), washed with PBS and dissolved, and radioactivity was measured by liquid scintillation counting. Protein synthesis was estimated from the sum of intracellular and extracellular radioactivity. Total protein content was determined by the Biorad protein assay (Bio-rad Laboratories GmbH, München, Germany) using bovine serum albumin as a standard.

Biochemical assays

Glycogen content was determined by quantitating glucose prior to and following hydrolysis of glycogen pool with amyloglucosidase (Boehringer Mannheim, Germany), using a glucose (Trinder) kit.

To measure cellular ATP, hepatocyte monolayers were first incubated with NRS (nucleotide releasing reagent for somatic cells, Lumac) to release ATP. Following appropriate dilution, the incubation medium was then used to measure ATP by a luciferin–luciferase assay.

Hepatocyte cultures containing 3×10^6 cells were used to determine the amount of reduced glutathione (GSH). After precipitation of cell proteins with metaphosphoric acid (MPA), GSH was measured in the supernatant using a colorimetric method (Bioxytech GSH-400 kit).

For each biochemical assay, the amount of protein was determined using the Biorad protein assay.

Statistical analysis

Data were expressed as mean $\pm$ SEM (standard error of the mean). Comparison between control group and treatment group was made by the Student's *t*-test for independent data. Differences were considered significant for p values < 0.05.

Results

Toxic effects of tacrine on hepatic cells

Rat hepatocytes in primary culture: In a first set of experiments, we used lactate dehydrogenase (LDH) release as a marker of the appearance of membrane alterations. This is illustrated in Figure 1, which shows the effects of different concentrations of THA on the extracelllular LDH/total LDH ratio in cultured rat hepatocytes. LDH release was estimated first after a 24 h treatment (Figure 1A) and then after a 48 h treatment (Figure 1B) with THA. From the dose–response curves, it appeared that THA elicited a dose-dependent increase of LDH release in cultured rat hepatocytes. Another interesting result was that a 0.1 mmol/L THA dose sufficed to induce a significant LDH release after a 48 h treatment compared to 0.15 mmol/L after a 24 h treatment, thus pointing to a time-dependent effect of THA. This was further confirmed when estimating the IC_{50}: 0.273 mmol/L for the 24 h treatment versus 0.129 mmol/L for the 48 h treatment.

In Figure 2, the incidence of the culture time on the appearance of THA toxicity toward hepatocytes was tested. To this end, rat hepatocytes were treated for 24 h with several con-

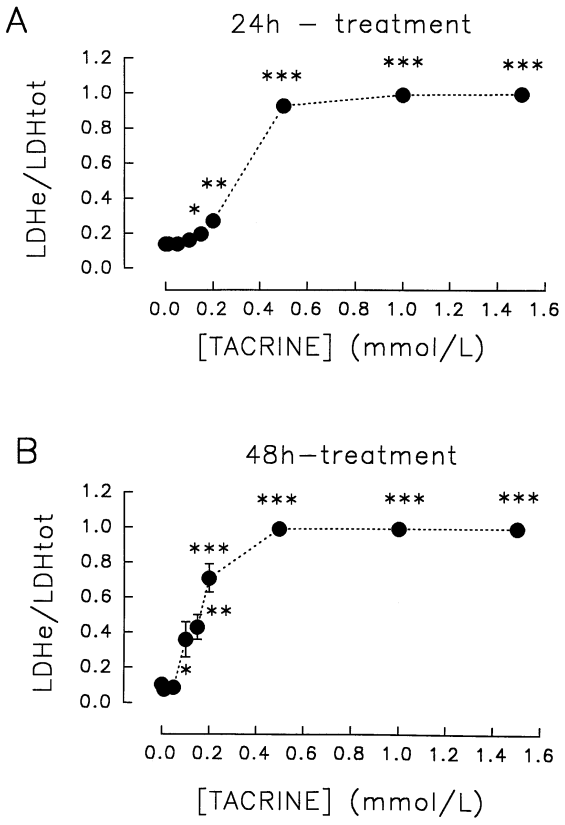

Figure 1. Effects of different concentrations of THA on the LDH release in rat hepatocytes in primary culture following a 24 h treatment (A) and a 48 h treatment (B). Values are the mean ± SEM of 3–5 independent experiments performed in triplicate. $*p<0.05$; $**p<0.01$; $***p<0.001$ (t-test; significantly different from untreated hepatocytes).

centrations of THA following different times in culture (i.e., 4, 24, 48 h after seeding). Whatever the time in culture, THA was found to be cytotoxic at 0.5 mmol/L (although a lesser degree of toxicity was observed for 48 h cultures). In contrast, at a dose of 0.25 mmol/L, only 4 h cultures were significantly altered (as indicated by a high LDH_e/LDH_{tot} ratio).

It has been speculated that bacteriostatic antibiotics such as streptomycin might promote, or conversely, attenuate, the hepatotoxic effects of THA (Fariss et al., 1994). Accordingly, rat hepatocyte cultures maintained in the absence of antibiotics were tested for the effects

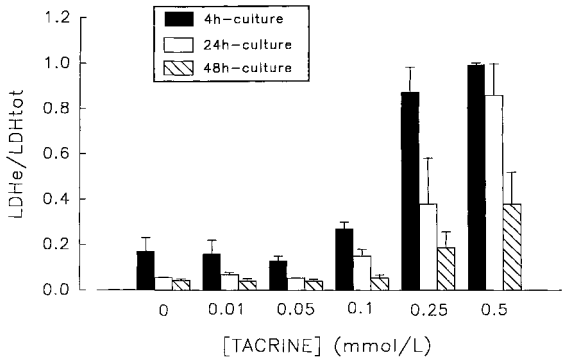

Figure 2. Influence of the time in culture of rat hepatocytes on THA cytotoxicity. Following different times after seeding, cultures were treated with tacrine for 24 h. Values are the mean ± SEM of 2 independent experiments performed in triplicate.

of THA. Under such conditions, the cytotoxicity of THA was unchanged (data not shown).

Rat liver epithelial cells: In order to further analyze a possible role for THA metabolism in the development of cytotoxicity, the effects of this molecule were tested on rat liver epithelial cells (RLEC), in which no expression of CYP 1A1/2 mRNAs was observed (Lerche et al., 1996). As shown in Figure 3, we first confirmed the absence of THA metabolism in such cells. Indeed, after 24, 48 or 72 h in presence of 0.1 mmol/L THA, only trace amounts (<0.5% of THA) of 1-, 2- and 4-hydroxy-THA could be detected in the culture media; in contrast, in hepatocytes, these metabolites amounted to about 47% of THA metabolism after 24 h of treatment. Despite this discrepancy in metabolic capabilities between rat hepatocytes and RLEC, Figure 4 shows that THA induced similar effects on LDH release, with a calculated IC_{50} of 0.278 mmol/L after a 24 h treatment (compared to 0.273 mmol/L in hepatocytes). Moreover, from Figure 3, it is notable that THA metabolism in rat hepatocytes decreased with time, unlike THA-induced cytotoxicity (see above).

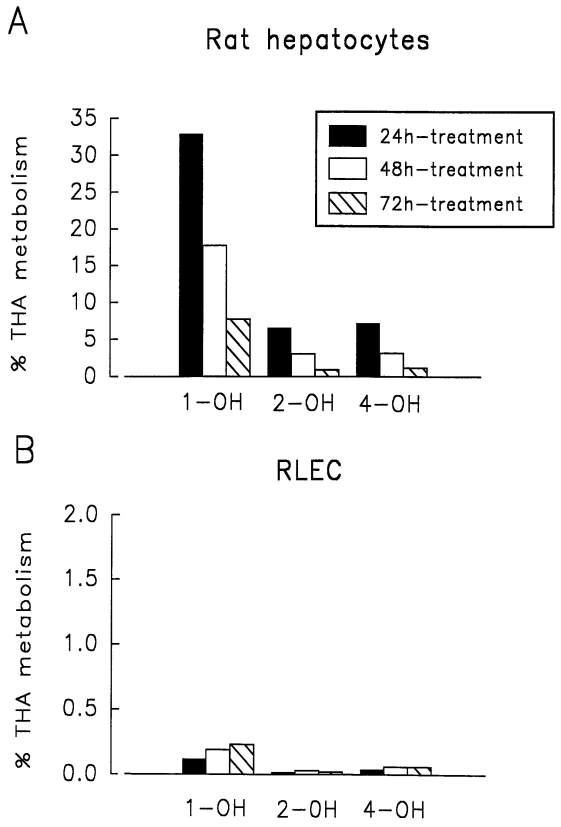

Figure 3. Formation of stable metabolites (expressed as percentage of detected THA) from THA in rat hepatocytes in primary culture (A) and in rat liver epithelial cells (B) following different times of treatment with 0.1 mmol/L THA. Note the different scale in ordinate between the two graphs. Values were obtained from a single experiment.

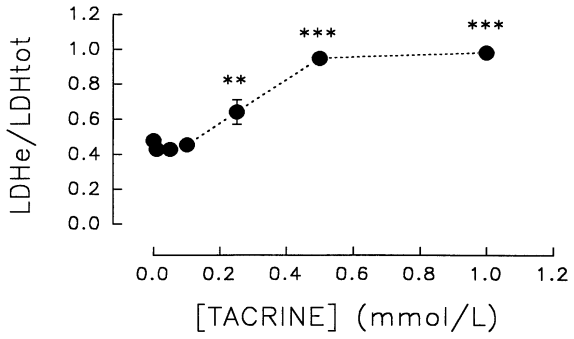

Figure 4. Effects of different concentrations of THA on the LDH release in rat liver epithelial cells following a 24 h treatment. Values are the mean ± SEM of 3 independent experiments performed in triplicate. $^{**}p < 0.01$; $^{***}p < 0.001$ (*t*-test; significantly different from untreated cells).

Table 1. Effects of a 24 h treatment with THA on the LDH release in human hepatocytes in primary culture

[THA] (mmol/L)	HL1 LDH_e/LDH_{tot}	HL2 LDH_e/LDH_{tot}
0	0.143 ± 0.006	0.120 ± 0.050
0.01	0.158 ± 0.020	0.112 ± 0.002
0.05	0.181 ± 0.010	nd
0.1	0.209 ± 0.017	0.139 ± 0.001
0.25	0.479 ± 0.012	0.352 ± 0.075
0.5	0.687 ± 0.045	0.406 ± 0.071
1	0.857 ± 0.032	0.625 ± 0.023
1.5	0.960 ± 0.043	0.990
IC_{50}	0.339 mmol/L	0.549 mmol/L

HL, human liver; nd, not done.

Each value represents the mean ± SEM of at least three samples from the same cell population.

Human hepatocytes in primary culture: Table 1 shows that THA also increased LDH release in human hepatocytes in primary culture. Nevertheless, after a 24 h treatment, slightly higher concentrations than those found in rat cells were needed to elicit a significant toxic effect as shown by the estimated IC_{50} (see Table 1).

Effects of tacrine on protein synthesis

Figure 5 illustrates the effects of a 24 h treatment with different THA concentrations on total (intracellular plus extracellular) protein synthesis estimated from rat hepatocyte cultures 4 h or 24 h after seeding. A significant decrease in protein synthesis was observed with 0.1 mmol/L THA when added 4 h after seeding, whereas a higher dose (0.25 mmol/L) was needed when cells were treated at 24 h. In one experiment, a significant decrease in protein synthesis was also observed in 0.1 mmol/L THA-treated rat liver epithelial cells following 3 days of treatment (data not shown).

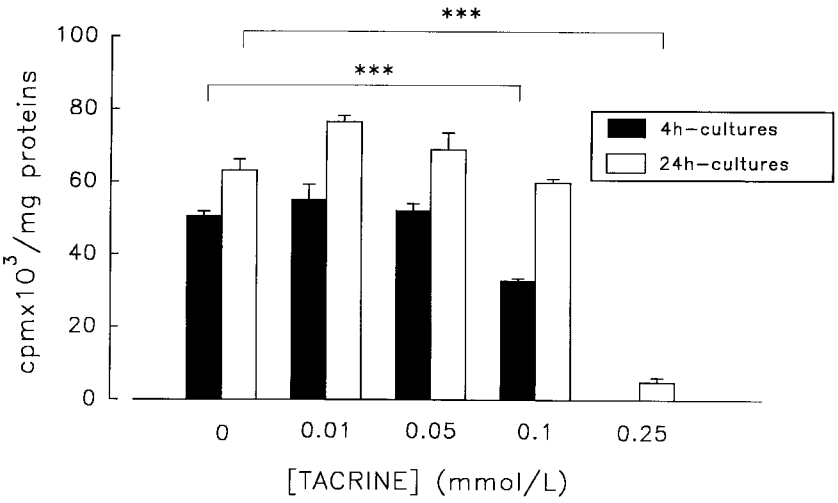

Figure 5. Effects of THA on protein (intracellular plus secreted) synthesis in rat hepatocyte in primary culture. The cells were maintained in the presence of varying concentrations of THA for 24 h. The influence of the time in culture of hepatocytes was also tested. Values are the mean ± SEM of 3 experiments. ***$p < 0.001$ (significantly different from untreated cells).

Effects of tacrine on biochemical parameters

In Figure 6, the effects of THA on both glycogen and intracellular ATP were tested. Following a 24 h treatment, whereas a significant decrease in glycogen level was observed at a THA concentration of 0.15 mmol/L (A), the ATP_i level remained unchanged even at doses as high as 0.25 mmol/L (B). In contrast, following a 48 h treatment (C and D), both parameters were significantly altered upon 0.1 mmol/L THA application, even though a significant decrease was actually observed at 0.075 mmol/L for glycogen level.

The effects of THA on reduced glutathione levels were tested following a 48 h treatment. Whereas in three independent experiments GSH content was unchanged at 0.1 mmol/L THA (19.6 ± 9.0 versus 16.3 ± 3.6 nmol/L per mg protein in untreated cells), a significant decrease (by about 5-fold; 3.5 ± 2.1 nmol/L per mg protein) was elicited by a THA dose of 0.2 mmol/L.

Effects of tacrine on cell ultrastructure

Figure 7 illustrates the rat hepatocyte ultrastructure in the absence (A) or presence (B) of THA. When short-term treatments (45 min to 2 h) with high doses of THA ($\geqslant 1$ mmol/L) were applied to the cells, aggregation of ribosomes and condensation of nuclear chromatin were then elicited. Similar alterations were obtained in rat liver epithelial cells (D). In human hepatocytes (C), condensation of nuclear chromatin was also present; moreover, in these cells, the endoplasmic reticulum structure was found to be altered with the appearance of electron-dense material in the dilated lumen of this organelle. Aggregation of ribosomes was also detected (not shown). At lower doses (0.05 and 0.1 mmol/L), even following 3 days of treatment, neither ribosome aggregation nor nuclear alteration could be detected in rat hepatocytes and liver epithelial cells; the mitochondrion did not appear to be markedly affected. The only alteration observed under these conditions was a reduction of glycogen particles in hepatocytes.

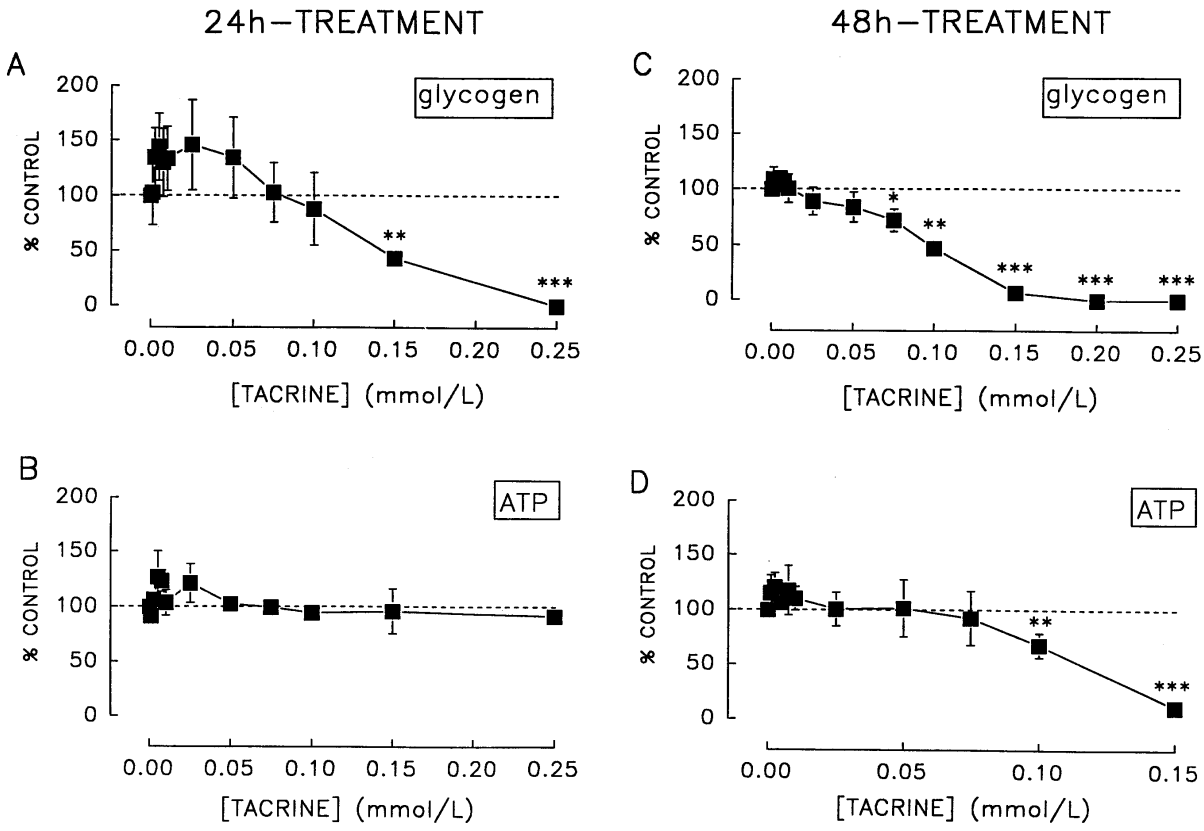

Figure 6. Glycogen and ATP levels in primary rat hepatocyte cultures after a 24 h treatment (A,B) and a 48 h treatment (C,D). Each point represents the percentage of glycogen or ATP level compared to control level estimated in untreated cells obtained from the same cell isolation. Values are the mean ± SEM of 4–5 independent experiments performed in triplicate. $*p<0.05$; $**p<0.01$; $***p<0.001$ (*t*-test; significantly different from untreated hepatocyte group).

Discussion

Nowadays, tacrine (THA) is currently used in the treatment of Alzheimer's disease. However, in about 30–50% of treated patients, an increase in serum liver marker enzymes (ALT) has been detected, pointing to the development of hepatotoxicity upon THA treatment. Despite the fact that the side-effects of THA have long been demonstrated, the exact mechanisms underlying such toxic effects still remain to be elucidated.

In the present study, the hepatotoxicity of THA was confirmed in rat hepatocytes in primary culture, and we have shown for the first time that liver epithelial cells are also susceptible to THA toxic effects. Therefore, our data indicate that both liver parenchymal and nonparenchymal cells are similarly affected by this molecule. This work also provides results from primary cultures of human hepatocytes. The toxic effects of THA on such primary cultures have recently been reported; however, only a short-term (4 h) treatment was tested (Monteith and Theiss, 1996). Here a longer treatment was applied (24 h) and it appeared that human hepatocytes in primary culture were somewhat less sensitive to THA than rodent liver cells, thus confirming data obtained in human hepatoma cell

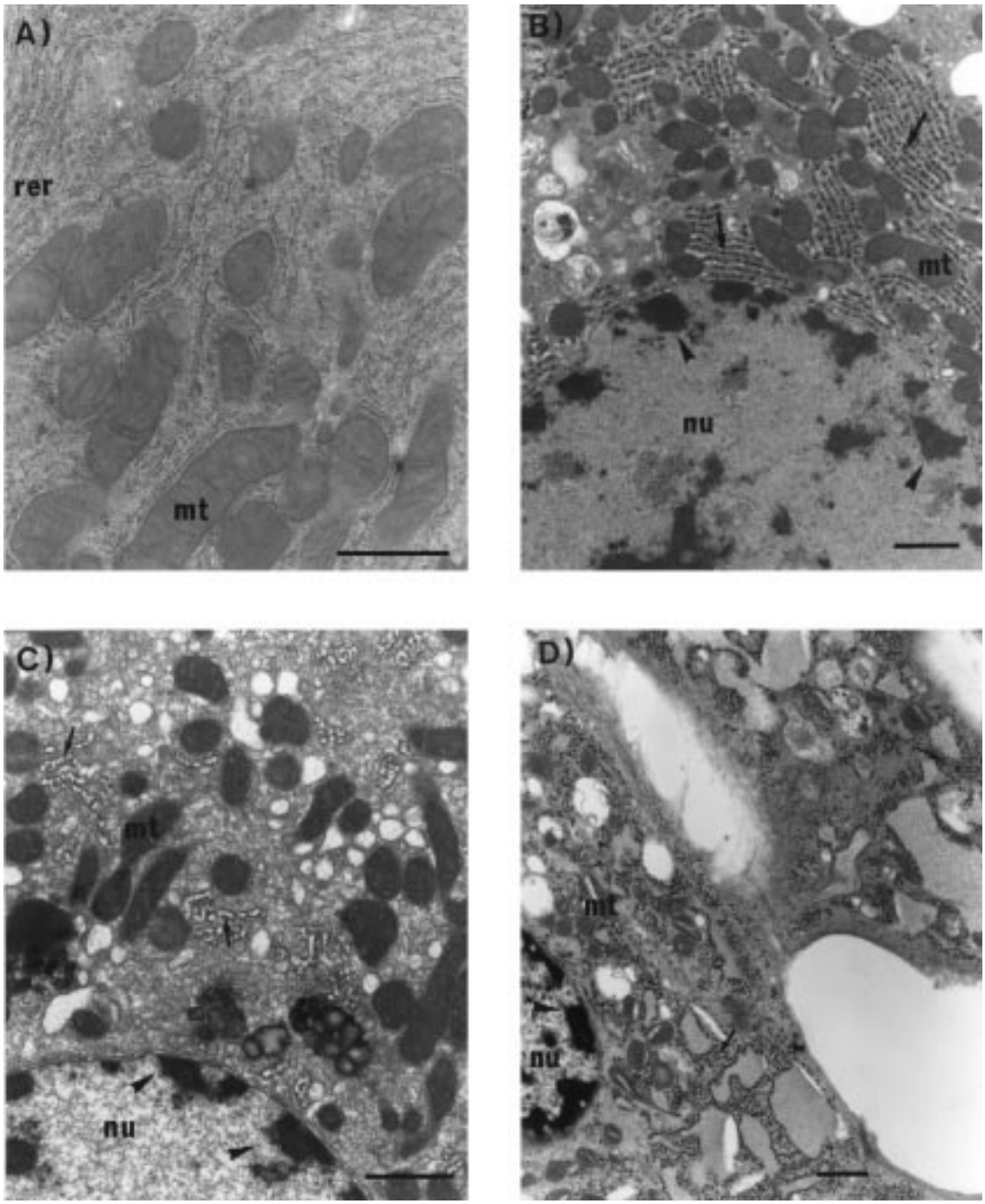

Figure 7. Effect of high doses of THA on cell ultrastructure. (A) Untreated rat hepatocytes in primary culture (magnification $\times 23\,800$; bar = 1 μm); rer: rough endoplasmic reticulum; mt: mitochondria. (B) Rat hepatocyte culture treated with 1 mmol/L THA for 1 h ($\times 9500$; bar = 1 μm). Note the condensed chromatin (arrowheads) in the nucleus (nu). Also note the aggregation of ribosomes (arrows). (C) Primary human hepatocyte culture treated with 1 mmol/L THA for 1 h ($\times 13\,200$; bar = 1 μm). Similarly to rat hepatocytes, condensed chromatin (arrowheads) is observed in the nucleus. Also note the dilatation of the rer (arrows). (D) Rat liver epithelial cells treated with 1 mmol/L THA for 1 h ($\times 7400$; bar = 1 μm). THA induces aggregation of ribosomes (arrows) and condensation of chromatin (arrowheads in the nucleus) in this cell type.

lines (Viau et al., 1993). The THA toxic effects have been found to be not only dose-dependent but also time-dependent, thus pointing to toxic effects related to accumulation of the molecule per se or one of its metabolites.

The hepatic microsomal cytochrome P450 enzyme system is known to play an important role in the metabolism of various xenobiotics, leading to the production of stable metabolites and, in some cases, of reactive metabolites. It is now well established that numerous drugs known as hepatotoxic cause injury via the production of this latter type of metabolite. With respect to THA, several studies have evidenced a major role for cytochrome P4501A2 in its metabolism (see Park et al., 1994; Balson et al., 1995 for reviews). Besides the production of stable metabolites such as 1-hydroxy-THA, metabolism of THA also results in the production of reactive metabolites, mainly quinone methide (Park et al., 1994). In this context, it has been suggested that the hepatotoxicity due to THA may result from the production of such a metabolite. In the present study, the fact that the THA toxicity was found to depend upon the time in culture of hepatocytes could have favored such a hypothesis. Indeed, our results showed that the toxicity decreased with time in culture and it has been reported that CYP level was decreased by about 50% after 24–48 h following cell seeding (Guzelian et al., 1977). Therefore, in 24 h and 48 h hepatocyte cultures, one might have supposed a less marked production of quinone methide, possibly underlying a reduced level of toxicity. However, two observations reported in this paper were against such a hypothesis: (1) THA toxicity increased with time of treatment (Figure 1) while the metabolic capacity of rat hepatocytes decreased (Figure 3A); (2) THA induced toxic effects in rat liver epithelial cells (in which THA metabolism was negligible; Figure 3B). With respect to this latter point, it was noteworthy that both rat hepatocytes and liver epithelial cells were similarly sensitive to THA toxicity, which might indicate the involvement of similar intracellular mechanisms. In support of a negligible role for THA-reactive metabolites is a recent paper by Benoit et al. (1997) showing that the V79 Chinese hamster cell line, not expressing cytochrome P450 activity, and its variants, genetically engineered for expression of human or rat CYP1A2, undergo similar toxicity upon THA treatment. Taken together, these results therefore point to a minor role, if any, of reactive metabolites in the hepatotoxicity of THA. Nevertheless, at present, one cannot completely rule out a role for THA-oxidative metabolism, especially as it has been shown in rat hepatocytes in primary culture that the toxicity of velnacrine maleate (i.e., the stable 1-hydroxy-THA metabolite) was markedly enhanced by GSH depletion and oxidative stress (Al Casey et al., 1995). In this context, the marked reduction in GSH content demonstrated in the present work at a dose of 0.2 mmol/L might further prompt the cells to die through the increased toxicity of this metabolite under such conditions. Furthermore, it is worthwhile noting that a role of GSTθ1 genetic polymorphism in THA toxicity in humans has recently been suggested (Becquemont et al., 1997).

Among the other factors possibly responsible for the toxic effects of THA, a role for ribosomal dysfunction has been put forward. With regard to such a role, Fariss et al. (1994) have shown that treatment with 1–2 mmol/L THA of freshly isolated rat hepatocytes in suspension resulted in a dramatic aggregation of ribosomes bound to the endoplasmic reticulum as well as in the aggregation of other nucleic acids both in the nucleus and in mitochondria. These electron-microscopic observations were associated with a rapid loss of cellular RNA content and a rapid and complete inhibition of protein synthesis, leading the authors to propose that the ribosomal dysfunction was responsible for these altera-

tions. In the present work, aggregation of ribosomes and nuclear chromatin condensation upon treatment with THA were also observed in all cells tested, that is, in rat hepatocytes in primary culture as well as in the rat liver epithelial cell line, with slightly different figures in human hepatocytes. However, as in the study quoted above, this was observed only at high doses ($\geqslant 1$ mmol/L). Therefore, it seemed unlikely that these ultrastructural alterations might have been responsible for the toxicity of THA observed at low doses following long-term treatment, or for the decrease in protein synthesis elicited upon treatment with a low, nontoxic dose (0.1 mmol/L) of THA. Indeed, as presently reported, such effects were observed in the absence of any ribosome aggregation or nuclear chromatin condensation in primary cultures of rat hepatocytes. Another argument against the involvement of ribosomal dysfunction in THA-induced hepatotoxicity was that THA elicited similar toxic effects in the presence or absence of bacteriostatic antibiotics (i.e., streptomycin) known to alter ribosomal function. Consequently, both ribosomal aggregation and nuclear chromatin condensation should be considered as nonspecific alterations related to an excess of THA and without any relevance to the *in vivo* situation. In support of this conclusion is our observation that THA doses >1 mmol/L also induce similar ultrastructural alterations in mouse 3T3 fibroblasts (not shown).

In a recent study it was reported that THA exerted a protonophoric effect in mitochondria, leading to a waste of energy and a decrease of adenosine triphosphate (ATP) in rat hepatocytes and human lymphocytes. This ATP depletion was shown to be the major cause of cell death in cells acutely exposed to high concentrations of THA (Berson et al., 1996). In the present study, an ATP depletion was also observed in THA-treated primary rat hepatocyte cultures. A significant decrease in ATP level was thus obtained following a 48 h treatment with a low dose (0.1 mmol/L) of THA. Under such conditions, THA was found to exert significant effects on glycogen level as well. However, our data indicated a greater sensitivity of glycogen level to THA as compared to ATP concentration. Indeed, following a 24 h treatment, 0.15 mmol/L THA induced a marked reduction (by $\sim 45\%$) of glycogen level while ATP level remained unaffected (even at the highest dose tested, i.e., 0.25 mmol/L); and a similar observation was obtained following a 48 h treatment with 0.075 mmol/L. The fact that glycogen level decreases prior ATP level may simply indicate that the cells utilize their glycogen stores in order to maintain normal intracellular ATP level as long as possible. Furthermore, it is worthwhile to note from the present data that the decrease in glycogen level at 0.15 mmol/L THA after a 24 h treatment was related to a significant increase (by $\sim 43\%$) in LDH release. Therefore, in our hands, it seems that primary rat hepatocyte cultures might undergo cell membrane alterations upon THA application without ATP depletion, thus suggesting a minor role, at least at early stages, for the previously reported THA-induced protonophoric effect on mitochondria (Berson et al., 1996). A likely explanation for such an observation might be that THA exerts direct effects on the cell membrane. In support of such a hypothesis, a recent study has demonstrated the preferential binding of THA to membrane acidic phospholipids (Lehtonen et al., 1996). Moreover, THA and velnacrine have been shown to strengthen cytoskeletal protein–protein interactions in erythrocyte membranes (Butterfield and Rangachari, 1992). Therefore, THA might partly exert its toxic effects via direct binding to membrane components.

In the present work, similarly to other toxic xenobiotics (see, e.g., Buc-Calderon et al., 1991), THA has been found to exert inhibitory effects on protein synthesis. This molecule might primarily affect the protein synthesis

machinery of the cell, even though one cannot exclude a THA-inhibiting action on a specific transport system for amino acids, via disturbances of membrane properties. It is also noteworthy that the decrease in protein synthesis has been observed before any significant release of LDH could be detected. This might suggest a role for this decrease in the appearance of cell death. In this respect, it is important to stress that other investigators have reported the ability of inhibitors of protein synthesis to induce and potentiate cell death in hepatocytes, mainly via apoptosis (Ledda-Columbano et al., 1992; Faa et al., 1994). Therefore, our data might suggest that hepatocytes permanently produce one or several proteins important for cell viability.

The effects of THA reported in the present study have been observed with doses about 100- to 1000-fold higher than those expected *in vivo* at the onset of the hepatic sinusoid (0.05–2.5 μmol/L; Berson et al., 1996). One might thus question the relevance of such direct effects of THA on hepatic cells in culture to the *in vivo* situation. At this stage, it is worth noting that a recent study describing a new *in vivo* animal model of THA-induced hepatotoxicity supports the hypothesis that this molecule, via an effect on the sympathetic nervous system, causes hypoxia in the liver, thus eventually resulting in hepatic necrosis (Stachlewitz et al., 1997). Therefore, according to that study, the origin of *in vivo* THA-induced hepatotoxicity would be indirect. However, it is important to outline several drawbacks in this model: (1) the dose necessary to induce this model was high (35 mg/kg) compared to that used in the treatment of AD patients (150 mg daily); and (2) hepatotoxicity developed after 12–24 h following THA administration and returned to control values by 32 h. In humans, however, transaminitis occurs after about 50 days of treatment; this may then allow more time for the accumulation of tacrine in the liver, and then more time for direct effects. Therefore, seeking *in vitro* the intracellular mechanisms underlying toxicity of THA would not seem inappropriate, especially as we have demonstrated the time dependence of THA effects.

In summary, the present study has shown that THA induces dose-dependent, toxic effects in both liver parenchymal and non-parenchymal cells. This toxicity is most likely independent of THA metabolism since similar effects were observed in rat hepatocytes and rat liver epithelial cells, in which THA metabolism was found to be negligible. Likewise, ribosomal aggregation is probably not involved in THA effects. Despite the fact that ATP depletion has been detected following THA treatment, this might be the result rather than the cause of cell death, since LDH release was found to increase before ATP level started to decrease. In contrast, glycogen level in hepatocytes appears to be a more sensitive indicator than ATP level of THA exposure and potential toxicity. Finally, as suggested by the experiments on protein synthesis, the decrease in synthesis of one or several still unknown proteins upon THA treatment might represent the signal leading the cells to death. Further experiments are now required to identify this protein or these proteins.

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