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Differences in cell proliferation in rodent and human hepatic derived cell lines exposed to ciprofibrate.

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Abstract

Peroxisome proliferators are widely used in different countries, especially in industrialized nations since they include hypolipidaemic agents of the fibrate family, plasticizers, herbicides as well as some solvents and pain additives. Human beings appear to be refractory to some effects of peroxisome proliferators activating peroxisome proliferator-activated receptor alpha (PPARalpha). Data from clinical trials and epidemiological studies do not demonstrate or rule out a human health risk associated with exposure to peroxisome proliferators. On the contrary rodents appear more susceptible to both carcinogenic and toxic effect of these peroxisome proliferators. In this study the possible differences between human and rat species has been evaluated examining the cell proliferative response to peroxisome proliferators by using rat and human tumour liver cell lines. A species difference in the response to peroxisome proliferators was observed. Rat 7777 hepatoma cells are more responsive than human HepG2 hepatoblastoma cells to ciprofibrate: 7777 cells show a higher decrease in cell number than HepG2 cells. Moreover, ciprofibrate is more toxic towards 7777 than to HepG2 cells: 7777 cells undergo apoptosis, whereas this does not happen to HepG2 cells. Oncogene c-myc is involved in both proliferation and apoptosis, but in opposite way where it decreases during proliferation reduction and increases during apoptotic process. Modulation of c-myc expression is controlled by PPARalpha as also demonstrated by increase lack of mRNA level in liver from Wy 14,643-treated PPARalpha-null mice. Moreover, the growth decrease is correlated with an increase of cells in G0/G1 phase, and a decrease of level of phosphorylated form of Erk1 and Erk2, whereas the of apoptosis increase with an increase of cells in S phase. These data provide evidence for a species difference between rodent and human liver cell lines in response to peroxisome proliferators. They are consistent with the idea that human cells are more refractory than rodent cells.

Key words : peroxisome proliferators; peroxisome proliferator-activated receptor alpha (PPARalpha), species difference; c-myc; human hepatoma cells; rat hepatoma cells, proliferation; apoptosis

1. Introduction

Numerous chemical molecules belonging to the peroxisome proliferators class of chemicals are present in the environment, increasing the risk of humans exposure [1,2]. Some of these chemicals are produced annually in high amounts by agro/pharma chemical industries, like plasticizers (adipates, phthalates), herbicides (chlorinated phenoxyacetates), paint solvents and anticorrosive additives (perfluoroacids). Fibrates are produced in smaller amounts but are frequently used as prescription drugs to prevent cardiovascular diseases decreasing serum lipids. Prolonged administration of peroxisome proliferators causes liver cancer in rodents [1], and the experimental evidence for this adverse effect in humans exposed to these chemicals is inconclusive [3]. In rodents, peroxisome proliferation is one indicator of exposure and possibly linked to the carcinogenic mechanisms since it may lead to an overproduction of intracellular H_2O_2 , a reactive oxygen species [4]. This event is mediated by PPAR α , an isotype of the PPAR family which is activated by these xenobiotics [5] and modulates the expression of various genes that regulate lipid metabolism including those involved in fatty acid beta-oxidation [6,7] and omega-oxidation (cytochrome P4504A). Anyway the mechanisms of peroxisome proliferator-induced hepatocarcinogenesis in rodents are not completely understood and could include alterations in cell proliferation and apoptosis (for recent reviews see, 8-11). To evaluate the potential human risk, molecular targets must be identified through experiments in animals as well as by using *in vitro* systems to elucidate the possible mechanisms. The use of *in vitro* systems revealed that peroxisome proliferators can be toxic in relation to the concentrations used. Several groups have shown that peroxisome proliferators at low concentration (0.05 mM) can suppress hepatocyte apoptosis that may normally act to remove damaged or excess cells from the liver [12-15]. Additionally, there is evidence that peroxisome proliferators at the same concentration induce increased replicative DNA synthesis and cell proliferation in rodent hepatocytes [16]. On the contrary, at higher concentrations than 0.05 mM peroxisome proliferators are toxic on tumour rodent cells, causing an increase of cells dead by apoptosis [17-19]. A comparison between rodent and human cell lines provides a good model to examine the molecular events induced by peroxisome proliferators to determine if there are species differences. For this reason in this study, *c-myc* oncogene expression and markers of cell proliferation were examined in rodent and human liver cell lines. The cellular oncogene *c-myc* encodes a nuclear transcription factor involved in both normal and

dysregulated cell division, and/or in apoptotic process. Previous work has shown that hepatic *c-myc* (or *N-myc*) mRNA level is increased in rodents treated with peroxisome proliferators [20-24]. More recently, it was reported that *c-myc* gene expression is dependent on the PPARalpha since PPARalpha null mice are refractory to induction of *c-myc* liver oncogene after treatment with a peroxisome proliferator [23]. Further support for a direct role of the PPAR in the regulation of *c-myc* gene expression was provided by the observation that mouse *N-myc* promoter contains a putative peroxisome proliferator response element (PPRE) [24]. PPARalpha highly expressed in the liver, other than in promoting fatty acid catabolism [25] is involved in several other functions, for example in cell cycle progression, in differentiation and death [26-27,28].

The goals of this study were: 1) to examine the effect of ciprofibrate, a peroxisome proliferator and hypolipideamic drug, on proliferation and death of tumour cell lines derived from human (HepG2 cells) or from rat liver (7777); 2) to estimate the correlation between *c-myc* and PPARalpha expression in hepatoma cell lines treated with ciprofibrate and in PPARalpha null-mice treated with Wy 14,643; 3, another potent peroxisome proliferator) to determine the effect of peroxisome proliferators on MAP kinase (Erk 1 and Erk 2) phosphorylated level.

2. Material and methods

2.1. Materials

Wy 14,643 and ciprofibrate (Figure 1) were purchased from Sigma (St. Louis, MO, USA), and Sanofi-Winthrop, respectively. Specific antibodies against PPARalpha, Erk 1 and 2 (phosphorylated and non-phosphorylated forms), and *c-myc* were purchased from Santa Cruz Biotechnology Inc. California. All culture media and reagents were purchased from Sigma.

2.1.1. Culture conditions and treatments

Rat hepatoma 7777 cells were seeded (20000 cells/cm²) and maintained in DMEM/F12 medium plus 2 mM glutamine, 1% antibiotic/antimycotic solution and 10% newborn calf serum. Human HepG2 hepatoblastoma cells were seeded (50000 cells/cm²) and maintained in MEM medium plus 2 mM glutamine, 1% antibiotic/antimycotic solution and 10% foetal bovine serum. 24 h after cell seeding, 7777 and HepG2 cells were treated with ciprofibrate, dissolved in DMSO (<0.05% final

volume), at concentrations of 0.05, 0.1, 0.20, 0.5 or 1 mM and harvested at 4, 24 and 48 h.

2.1.2. PPARalpha-null mice

PPAR alpha (-/-) male mice on an Sv/129 background, generated according to Lee et al, 1995 were housed 4-5 animals per cage in a temperature -and light controlled environment (T=25°C, 12h light/dark cycle). Wild and PPARalpha null mice were fed with or without 0.1% Wy-14,643 for 2 weeks

2.1.3. Cell proliferation

Cell proliferation was evaluated as the number of cells present in the monolayer and in the culture medium. Cell numbers were determined by using a Bürker chamber.

2.1.4. Lactate dehydrogenase activity

Lactate dehydrogenase activity (LDH) was determined in the culture medium by spectrophotometric assay and was expressed as nmoles of NADH consumed/ml of medium [29].

2.1.5. Fluorescence microscopy

Cells were fixed in methanol, stained with the DNA-specific fluorochrome DAPI and examined under an epiilluminated fluorescence microscope (Dialux, Leitz, Germany). The apoptotic index (percentage of apoptotic cells) was determined on at least 1000 cells per sample.

2.1.6. DNA distribution

The percentages of cells in different phases of the cell cycle and the percentage of cells present of subG0/G1 peak were evaluated by flow cytometry determining the DNA content after propidium iodide staining, as described elsewhere [30]. The percentages are calculated respect to total counted cells.

2.2. Northern Blotting

After RNA extraction, northern blotting was performed using appropriated ³²P labelled probes (using random priming method). The human c-myc cDNA (Oncor product) for human RNA hybridizations, murine c-myc cDNA [31] for mouse RNA hybridizations, mouse peroxisomal acyl-CoA oxidase cDNA for all blots [20], and human β-actin cDNA as a control for all blots. Autoradiographs were scanned with a densitometer and relative hybridisation signals were calculated based on the percentage of the beta-actin signal.

2.2.1. Western Blot analysis

Cellular extracts were prepared as described in [31] and blotted with polyclonal anti-PPARalpha, anti-pErk 1,2 (phosphorylated form), anti Erk 1,2 (unphosphorylated form) or anti-c-myc antibodies. For determination of the content of PPARalpha protein in HepG2, immunoprecipitation was used.

2.2.2. Protein determination

Protein content was determined by the Protein Assay Kit 2 (Bio-Rad Laboratory, Inc.).

2.2.3. Statistical analysis

All data were expressed as means \pm S.D. Significant differences between group means were determined by analysis of variance, followed by the Newman-Keuls test.

3. Results

3.1. Cell growth of human and rat hepatoma cells exposed to ciprofibrate.

The effect of ciprofibrate on human HepG2 hepatoblastoma and rat 7777 hepatoma cell proliferation was examined. The former cells exhibited a decrease in proliferation upon culture in 0.25 mM ciprofibrate (Fig. 2A) starting from 24 h treatment, whereas this effect was not observed at the lower concentrations. To compare this inhibitory effect found in human HepG2 cells with rat hepatoma cells, the effect of ciprofibrate on cell proliferation was examined in rat 7777 hepatoma cell line (Fig. 2B). Cell proliferation was significantly reduced as a result of ciprofibrate treatment in this rat hepatoma cell line, and this effect was more pronounced compared to HepG2 cells in the presence of the highest concentrations, especially at 48 h treatment. For example with 1 mM ciprofibrate at 48 h exposure, inhibition of cell proliferation was 68% for rat 7777 hepatoma cells, whereas it was only 43% for HepG2 cells. Inhibition of HepG2 cell proliferation by ciprofibrate was not due to cytotoxicity, as detached cells were not found in the medium, and lactate dehydrogenase activity in the medium was identical to that of the control cells (data not shown). Moreover, the presence of apoptotic cells, as determined by staining the cells with DAPI and by examining them with flow

cytometry (Table 1), showed very low percentage after 48 h exposure (for HepG2 cells). In contrast, for rat 7777 hepatoma cells at 48 h exposure there was 10% of cells detached from the monolayer, and all detached cells were undergoing apoptosis, as determined by evaluating the presence of sub G0/G1 peak by flow cytometry. Also in the monolayers of 7777 cells exposed for 48 h to 0.5 or 1 mM ciprofibrate, apoptotic cells were present and their percentages were 29 and 37%, respectively (Table 1). These results confirm that human HepG2 cells are more resistant to ciprofibrate than rat hepatoma cells. In fact, ciprofibrate is not cytotoxic on HepG2 cells, whereas it is on rat hepatoma cells.

To identify the phase of the cell cycle that was changed in response to ciprofibrate, the DNA content was determined by flow cytometry in HepG2 and in 7777 cells after treatment with. Results from this analysis indicate that in HepG2 cells ciprofibrate decreases cell growth by causing a block in the G0/G1 phase, and a decrease in the cells entering the S phase (Fig.3), whereas in 7777 cells it increases the percentage of cells counted in S phase, more markedly at 48 hours (Fig.4).

Consistent with the decrease of cell growth, in HepG2 cells there is a decrease in phosphorylated form of Erk (a MAP kinase subunit and a key component of the mitogen signaling pathway), without changing the total Erk 1,2 protein content (Fig. 5).

3.2. *c-myc* and *PPARalpha* gene expression in human and rat hepatoma cells and in *PPARalpha*-null mice

Due to the role played by *c-myc* oncogene, in the cell proliferation control our interest, in studying the modulation of *c-myc* level in hepatoma cells treated with peroxisome proliferators, prompted us to examine the correlation between *c-myc* and *PPARalpha* expression both *in vitro*, in human and rodent hepatoma cell lines, and *in vivo*, in *PPARalpha*-null mice. In human HepG2 cells, ciprofibrate decreases both *c-myc* and *PPARalpha* protein content at all experimental times considered (Fig.6A), being the more marked reduction evident in *c-myc* content at 48 h exposure (inhibition percentage 55%). In rat 7777 cells (Fig.6B), ciprofibrate shows opposite effect at 24 or 48 h exposure; in fact, at 24 h a strong reduction of both *c-myc* (- 75%) and *PPARalpha* (- 45%) protein occurs, whereas at 48 h *c-myc* protein increases almost three times and *PPARalpha* more than eight times.

Fig. 7A shows that treatment with Wy114,643 (another peroxisome proliferators with similar effects than ciprofibrate) for 10 days results in a 4-fold increase of *c-myc* mRNA in wild-type mice compared to untreated wild type mice, whereas this effect is

largely diminished in PPARalpha-null mice. As positive controls hepatic levels of mRNA for PPARalpha and peroxisomal AOX are induced by Wy 14,643 in wild-type mice, and this effect is absent in PPARalpha-null mice (Fig7B).

4. Discussion

Since the mechanisms underlying the putative species differences in the response to peroxisome proliferators are not well characterized, in this study, a comparative molecular analysis on hepatic derived cells of both human and rodent species was performed.

4.1. Cell growth

Significant species differences in the effect of peroxisome proliferators on liver mass is also known to exist [32,33]. By measuring the hepatosomatic index of three species treated at a dose of ciprofibrate resulting in the same plasma level of the drug, (thus at the pharmacoequivalence to tissues), an increase in liver weight is only observed in the treated rat, while no significant change in liver weight is found in the jerboa [34] or guinea pig [35]. Our *in vitro* study comparing the responsiveness of human and rat hepatoma cells to ciprofibrate also showed differences: the growth of both types of cells from different species is negatively regulated by ciprofibrate, but less in human hepatoma cells. The more marked reduction of cell number occurring in rat cells is evident in presence of 0.5 and 1 mM ciprofibrate at 24 and 48 h exposure. Moreover, in the human cells the reduction of cell number is not due to the appearance of cell death, like as in rat hepatoma cells, where a massive apoptosis occurs at 48 h exposure in presence of 0.5 mM ciprofibrate. The susceptibility to apoptosis showed by rat 7777 hepatoma cells is in agreement with previous reported data on another rat hepatoma cell line, Fao cells. In fact, these cells exposed to ciprofibrate and analysed by flow cytometry show modifications of intrinsic cellular parameters, such as size and cytoplasmic granularity, indicating the occurrence of apoptotic death. In the same study in ciprofibrate-treated HepG2 cells no apoptosis was observed [36]. It is to note that in this previous study the concentration of ciprofibrate used was not up 0.2 mM; whereas in the present study higher concentrations were used.

A different response between human and rat tumour cells is also evident in the distribution of cells in the different phases of cycle. In fact, in HepG2 cells the reduced cell growth corresponds to an increased population in G₀/G₂ phase; whereas in 7777 cells the induction of apoptosis correspond to an increased percentage of cells in S phase. We observed that also in presence of clofibrate, another peroxisome proliferators, 7777 cells are blocked in S phase and undergo apoptosis (unpublished

data). In the same way, a previous study on the cell cycle distribution of asynchronously growing rat Fao cells showed an increase in the percentage of proliferative cells in ciprofibrate-treated cells [37].

4.2. Correlation between c-myc oncogene expression and PPARalpha

It is well known that oncogenes are involved in some effects of peroxisome proliferators. The oncogenes c-myc or N-myc encoding for transcription factors have an essential role in the rodent carcinogenic process involving peroxisome proliferators [20, 37, 21, 38]. Other oncogenes may also be involved [16,20,37,21,39,38] as well as key proteins of the cell cycle [40]. We have shown [24] that overexpression of c-myc occurs in liver of rats treated with either clofibrate or ciprofibrate for a short (two weeks) or a long duration of time (one year). This effect is relatively liver specific since only a small c-myc mRNA level increase is found in kidney and no effect is observed in brain or heart [20].

In this study, we show that in HepG2 hepatoblastoma cells c-myc expression is negatively regulated by ciprofibrate at all experimental times considered, whereas in rat 7777 hepatoma cells, the regulation is negative during the first 24 h exposure, but becomes positive at the next experimental time. This different effect during the time could be related to the fact that in the first 24 h exposure 7777 cells undergo only a decrease in cell proliferation, whereas in the following time they show apoptotic death. The c-myc variations in both human and rat cell lines show the same behaviour than those of PPARalpha.

The transcription of some oncogenes might be PPAR dependent, since several oncogenes have a putative PPRE [24], in particular the mouse N-myc which shows an almost canonic sequence at the promoter region, as well as at the c-sis (related to beta-chain of PDGF) upstream sequence. Some PPRE-related sequences have also been found in other genes encoding proteins involved in carcinogenesis or cell proliferation: human v-raf, human and mouse p53, human c-fos, rat carcinogenic antigen, human c-fms or chicken TGFbeta 3 gene [24]. Here, we show that the stimulation of c-myc expression by peroxisome proliferators is dependent on PPARalpha since the stimulation is largely abolished in liver from Wy 14,643-treated PPARalpha-null mice, which is consistent with a previous report [41]. Enhancement of c-myc expression is also increased in AOX-null mice that spontaneously develop liver tumours (unpublished observation), which is of interest since PPARalpha expression is higher compared to wild-type animals [42, 43]. Moreover, in this research it has been shown that, at high

concentrations of ciprofibrate (>0.5 mM), induction of apoptosis is associated with c-myc and PPARalpha increased expression, whereas inhibited cell proliferation is associated with slightly lower PPARalpha and c-myc expression.

4.3. Species difference

The mechanisms underlying the species difference might be due to the lack or to different intermediate metabolic steps in non-responsive species. For example, it is likely that there are differences in xenobiotic metabolism and pharmacokinetics between highly and weakly responsive species. Indeed, ciprofibrate is cleared at a much higher in guinea pig than in rat [35]. The species differences might also be due in part to relatively lower levels of hepatic PPARalpha which is very low in liver cells of non-responsive species when compared to rat and mice the high response species [44]. In rat, the PPARalpha tissue distribution shows the highest expression in liver as compared to other tissues [45] which is consistent with the lack of tumor formation in extrahepatic tissues of rodents triggered by peroxisome proliferators. In the human and rat cultured cells we used there is also difference in the content of PPARalpha; in fact, in HepG2 cells immunoprecipitation needed to be used to evidence it. The less content of PPARalpha protein could explain the more resistance of HepG2 to toxic effect of ciprofibrate.

In conclusion, our present work reports a species difference response of c-myc expression after peroxisome proliferator exposure at the last experimental time; moreover, we confirm that modulation of c-myc expression by peroxisome proliferators is dependent on PPARalpha, since its stimulation is partially abolished in liver from Wy 14,643-treated PPARalpha-null mice. In a previous report, Peters et al [41] demonstrated that c-myc activation is completely dependent on the PPARalpha since PPARalpha-null mice were refractory to the increase of this mRNA after exposure to Wy 14,643 for either 5 weeks or 11 months. In the present study, exposure to Wy 14,643 was limited to 10 days.

The resistance of human HepG2 cells towards peroxisome proliferators is explained by the different response to ciprofibrate in comparison with rat cells. At the present time of knowledge, our data are consistent with the idea that peroxisome proliferators represent a low, or no risk to humans.

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7. Legend of figures

Figure 1: Chemical structures of ciprofibrate and Wy 14,643

Figure 2: Effect of ciprofibrate on cell proliferation of hepatic derived cell, human HepG2 (A) and rat 7777 cells (B). Values are means \pm S.D. of 3 experiments. Ciprofibrate was dissolved in DMSO, at concentrations of 0.05, 0.1, 0.25, 0.5 or 1 mM. Cells were exposed to ciprofibrate for 4, 24 and 48 hours.

Means with different letters are statistically different ($P < 0.001$) from one another as determined by ANOVA followed by the Newman-Keuls test.

C: control cells

Cipro: cells exposed to ciprofibrate at the indicated concentrations

Figure 3: Effect of ciprofibrate on the cell cycle phases in human HepG2 cells. Values are expressed as percentages of cells present in the G_0/G_1 , S and G_2/M phases, and are means \pm S.D. of 3 experiments. S.D. (not reported in the figure) are less than 10%.

C: control cells

Cipro: cells exposed to ciprofibrate at the indicated concentrations (in mM)

Figure 4: Effect of ciprofibrate on the cell cycle phases in rat 7777 cells. Values are expressed as percentages of cells present in the G_0/G_1 , S and G_2/M phases, and are means \pm S.D. of 3 experiments. S.D. (not reported in the figure) are less than 10%.

C: control cells

Cipro: cells exposed to ciprofibrate at the indicated concentrations (in mM)

Figure 5: Effect of ciprofibrate (Cipro) on pErk1,2 and Erk1,2 level on human HepG2 cells as revealed by western blot analysis.

Indications on treatment (dosage and length) are given in the results of the figure

Figure 6: Effect of ciprofibrate (Cipro) on c-myc and PPARalpha level on human HepG2 (A) and rat 7777 (B) cells as revealed by western blot analysis, excepted to evidence the content of PPARalpha in HepG2 the immunoprecipitation method was used, since the Western Blot was not enough sensible.

Indications on treatment (dosage and length) are given in the results of the figure

Figure 7: Northern blot analysis of liver c-myc (Fig. 7A), AOX and PPAR α (Fig. 7B) mRNA expression in wild-type (+/+) and PPARalpha-null (-/-) mice. Fold increase in presence of Wy 14,643 (Wy) represents increase in relative hybridization signal (% beta-actin) as compared to respective control (C). The blot is representative of three different blotting

8. Tables

Table 1 - Percentage of apoptotic cells in human and rat hepatoma cells after treatment with ciprofibrate.

CELLS	NONE		0.5 mM CIPROFIBRATE		1 mM CIPROFIBRATE	
	24 h	48 h	24 h	48 h	24 h	48 h
HepG2	3.41 \pm 0.35a	3.02 \pm 0.26a	2.82 \pm 0.40a	2.56 \pm 0.33a	2.99 \pm 0.45a	7.09 \pm 0.56b
7777	0.39 \pm 0.09a	6.52 \pm 0.76b	4.89 \pm 0.67b	29.42 \pm 2.77c	7.01 \pm 1.89d	36.99 \pm 4.91c

Data obtained from flow cytometry analysis are expressed as percentage of apoptotic cells respect to total cells and represent means \pm S.D. of three experiments.

Human HepG2 hepatoblastoma cells were seeded (50000 cells/cm²) and maintained in MEM medium plus 2 mM glutamine, 1% antibiotic/antimycotic solution and 10% foetal bovine serum. Rat hepatoma 7777 cells were seeded (20000 cells/cm²) and maintained in DMEM/F12 medium plus 2 mM glutamine, 1% antibiotic/antimycotic solution and 10% newborn calf serum. 24 h after cell seeding, HepG2 and 7777 cells were treated with ciprofibrate, dissolved in DMSO, at concentrations of 0.5 or 1 mM and harvested at 24 and 48 h.

Figure 1

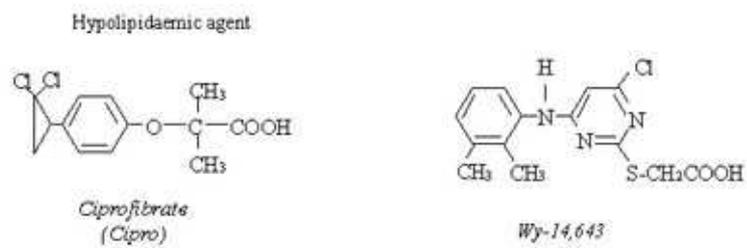


Figure 2

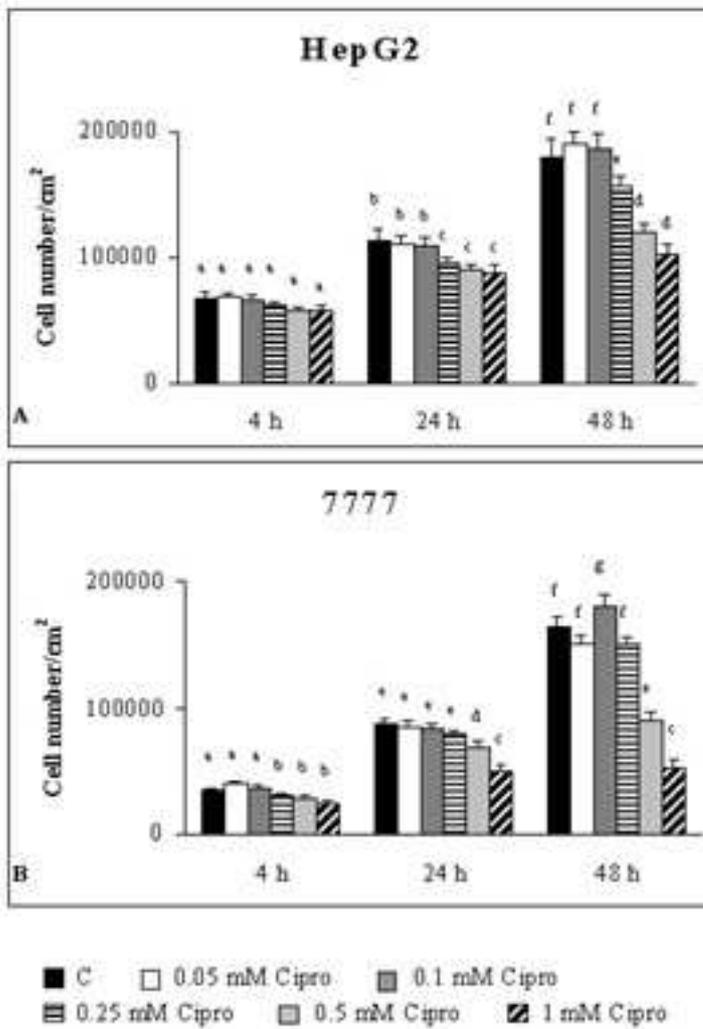


Figure 3 - Hep G2 hepatoma cells

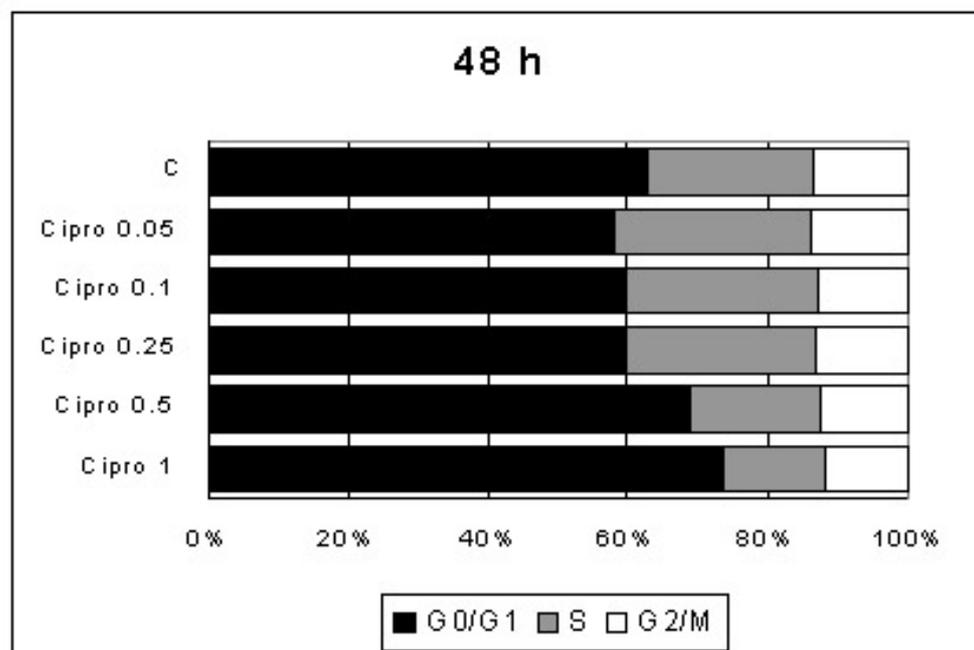
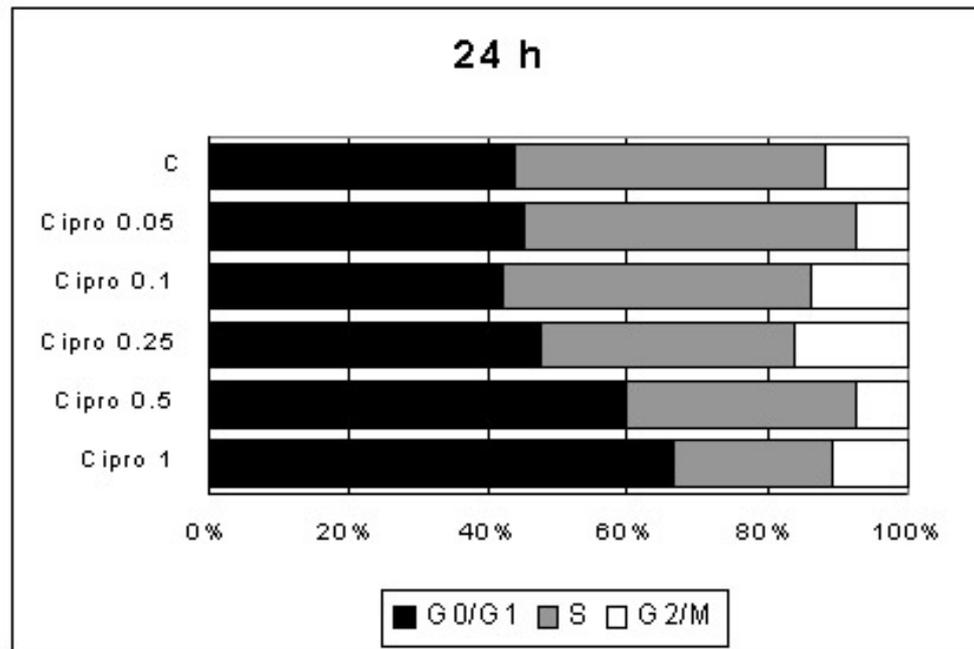


Figure 4 - 7777 hepatoma cells

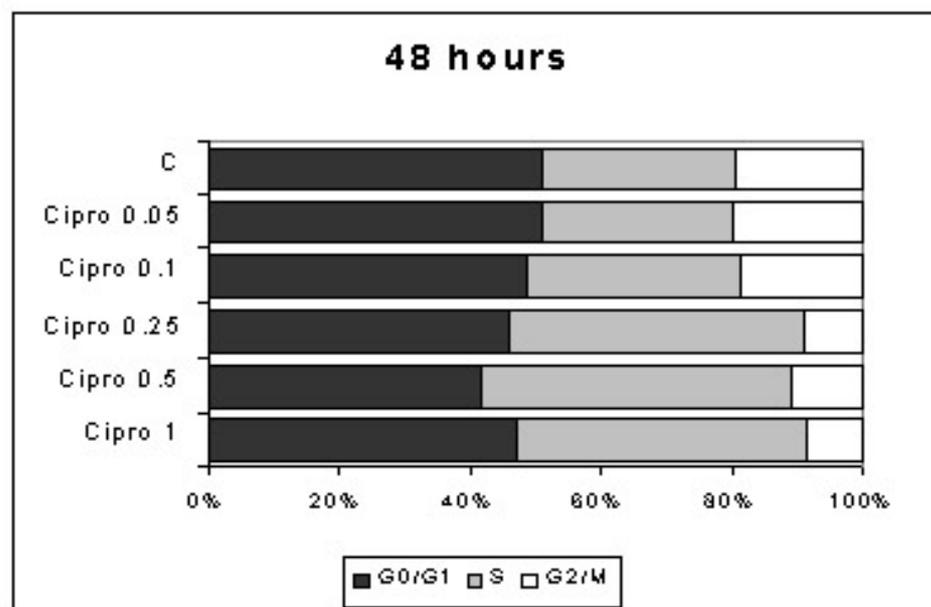
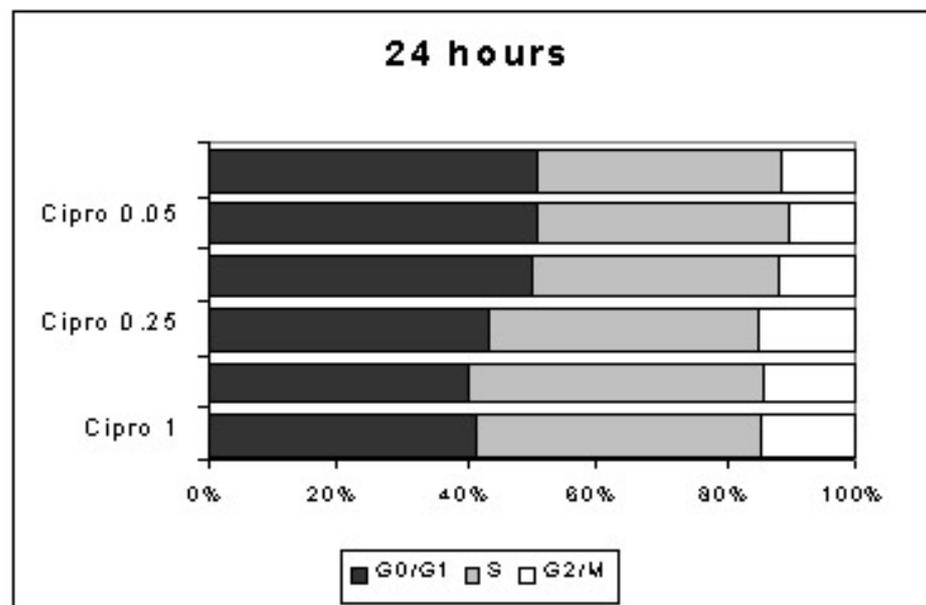


Figure 5

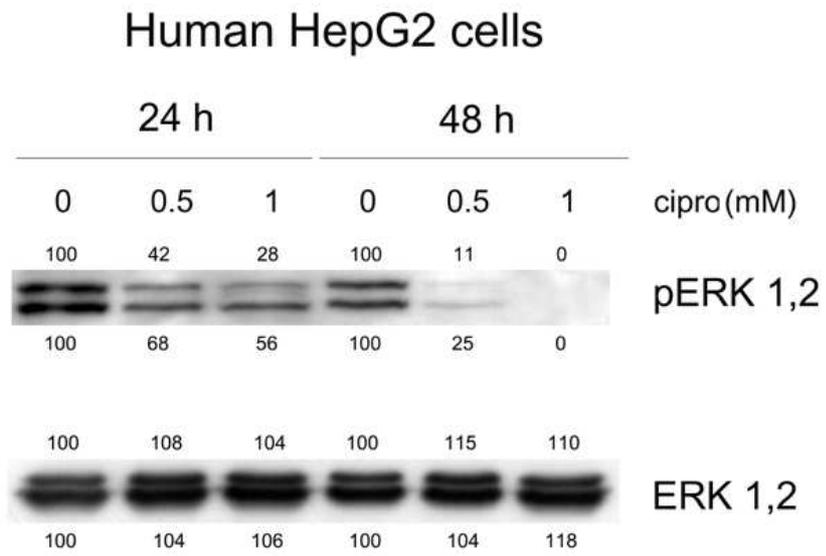


Figure 6

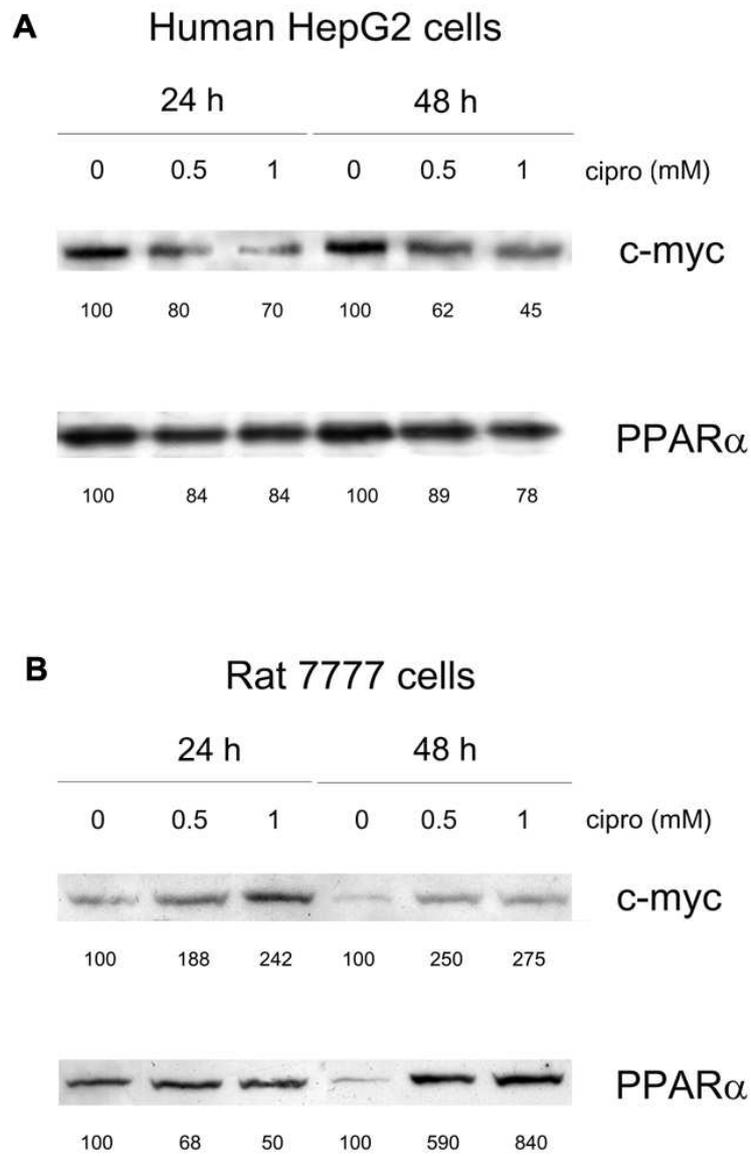


Figure 7

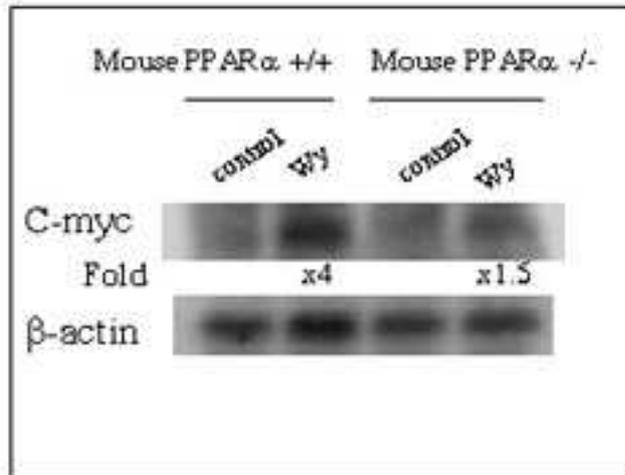


Figure 7

