Apoptosis induced by clofibrate in Yoshida AH-130 hepatoma cells: role of HMG-CoA reductase

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Abstract Clofibrate is a hypolipidemic drug belonging to the peroxisome proliferator (PP) family. PPs are well-recognized hepatocarcinogens, though only for rodents and not for humans. Their oncogenicity is usually ascribed to mitogenic or antiapoptotic action. However, we have reported that clofibrate can trigger fast and extensive apoptosis in rodent and human tumor cell lines. The present study examines the possible mechanisms involved in clofibrate-induced apoptosis in AH-130 hepatoma cells. The results show that the apoptogenic effect of clofibrate does not depend on induction of peroxisome proliferator activated receptors (PPARs), but on interference with HMG-CoA reductase (HMGR), a key enzyme that regulates cholesterol biosynthesis and production of isoprenoid units for protein farnesylation. The level and activity of HMGR mRNA are reduced in clofibrate-treated AH-130 cells and apoptosis can be partially prevented by addition of mevalonate. Moreover, cholesterol and cholesterol ester content decreases early in mitochondria, and cytocrome c is released in the cytosol. On the contrary, perturbations at the level of protein farnesylation are not important in determining the fast apoptogenic effect, since treatment of AH-130 cells with an inhibitor of farnesyltransferase induces apoptosis only after 4 h. In conclusion, inhibition of HMGR and decreased cholesterol content are crucial events in clofibrate-induced apoptosis in AH-130 hepatoma cells.—Canuto, R. A., G. Muzio, M. Maggiora, A. Trombetta, G. Martinasso, R. Autelli, P. Costelli, G. Bonelli, and F. M. Baccino. **Apoptosis induced by clofibrate in Yoshida AH-130 hepatoma cells: role of HMG-CoA reductase.** ***J. Lipid Res.*** **2003.** 44: **56–64.**

Supplementary key words cholesterol • hydroxy-methyl-glutaryl coenzyme A reductase • peroxisome proliferator activated receptors • mevalonate

Peroxisome proliferators (PPs) are well-recognized hepatocarcinogens for rodents, though nor for humans (1–3). Generally categorised as non-genotoxic carcinogens, their oncogenicity is mostly ascribed to their capability to alter the balance between liver cell proliferation and apoptosis during the transformation process. In rats, indeed, PPs both induce hepatocyte proliferation and decrease apoptosis in preneoplastic liver lesions (4, 5) and, consistently, they promote mitogenesis as well as survival in rodent hepatocytes or hepatoma cells in vitro (6, 7). By contrast, in primary cultures of human hepatocytes, none of several PPs afforded any preventive action against on apoptosis, whether spontaneous or TGFβ1-induced (8, 9).

The effects of PPs are mediated by members of the nuclear hormone receptor superfamily known as PP-activated receptors (PPARs), which exist in different molecular forms (α, β/δ, and γ) that are differentially expressed in adult and embryonic rodent tissues (10). Upon PP binding, PPARs recognize specific PP-responsive DNA elements (PPREs) and thereby transactivate a number of genes, including those involved in peroxisome proliferation and fatty acid β-oxidation or protooncogenes such as c-*Ha-ras,* c-*myc,* and c-*Jun* (11, 12). The induction of peroxisomal enzymes can result in overproduction of reactive oxygen species that may initiate lipid peroxidation and stimulate apoptosis in different cell types (13). In virtue of this induced free radical generation, PPs might exert some genotoxicity on target cells, which has been suggested as an alternative or additional mechanism accounting for their oncogenic action. PPARα is the most abundant isoform in the liver and is thought to mediate the carcinogenic action of PPs in rodents. Factors such as presence of a truncated PPARα form, scarcity of full-length PPARα, or occurrence of inactive PPRE consensus regions upstream of target genes such as acyl-CoA oxidase have been suggested to account for the unrespon-

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Abbreviations: DAPI, 4′,6′-diamidino-2′-phenylindole; HMGR, hydroxy-methyl-glutaryl coenzyme A reductase; PP, peroxisome proliferator; PPAR, peroxisome proliferator activated receptor; PPRE, peroxisome proliferator response element.

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siveness of human liver or hepatoma cells to these agents (14–16).

Like other fibric acid derivatives, the hypolipidemic drug clofibrate is a well-known PP that, though never proven to cause human tumors, has been widely employed in hepatocarcinogenesis protocols for rodents (1, 17). Like other PPs, the carcinogenicity of clofibrate has been largely ascribed to its antiapoptotic action. However, we fortuitously observed that, in vitro, clofibrate can also directly trigger an extensive and quick process of apoptosis in rat Yoshida AH-130 hepatoma cells (18, 19). Moreover, we found that this unexpected apoptotic effect was also exerted on HepG2 human hepatoblastoma cells (18, 19). These initial observations have since been extended to other PPs and cell types. Thus, Shabalina et al. (20) reported that another potent PP in rodents, perfluorooctanoic acid, can perturb the proliferative cycle and trigger apoptosis in human HepG2 cells. Moreover, both human (HepG2) and rat (FAO) hepatoma cells apoptose when exposed to BR931, yet another hypolipidemic drug endowed with PP properties (A. Columbano, personal communication). Apart from cells of hepatic lineages, wherein PPARα is thought to play a major role in mediating PP actions, apoptosis has been observed in various other cell types, such as human and mouse breast cancer cells or human lung cancer cells, on exposure to various synthetic or natural ligands of another member of the PP-receptor family, PPARγ (21, 22).

Starting from our observation that clofibrate exerts an apoptotic effect on Yoshida AH-130 rat hepatoma cells, we decided to seek the mechanisms underlying this effect. The paper examines two principal ways: involvement of PPARs and involvement of HMG-CoA reductase (HMGR). This second way is considered since clofibrate is a hypolipidemic drug as well as a PP; therefore, it might exert its apoptotic effect not only by activating PPARs, like other PPs, but also by inhibiting HMGR. HMGR is a key enzyme in cholesterol and isoprenoid synthesis, and it is known that its activity and protein content are affected by PPs (23). The possibility that clofibrate-induced apoptosis may result from inhibition of HMGR leads us to consider the importance of decrease of cholesterol content or isoprenoid production. Isoprenoids are involved in farnesylation of proteins important for cell cycle progression (24–26). Our previous results evidenced that cholesterol content increases in mitochondria of hepatoma cells, altering the physical and functional properties of the membranes (27, 28).

MATERIALS AND METHODS

Chemicals

4′,6′-Diamidino-2′-phenylindole (DAPI) was from Boehringer Mannheim (Germany); clofibrate [2-(p-chlorophenoxy)-2 methyl-propionic acid ethyl ester], DNase-free RNase (Type II-A), propidium iodide, and the other reagents were from Sigma (St. Louis, MO). Monoclonal anti-Ras antibody was from Cal Biochem. Monoclonal anti-cytochrome c antibody was from BD PharMingen.

Cells and treatments

Male Long-Evans rats, weighing 120–150 g and maintained on a semi-synthetic diet (Piccioni, Brescia, Italy) with water ad libitum, were used for serial weekly transplantation of the AH-130 Yoshida ascites hepatoma ($20–30 \times 10^6$ tumor cells inoculated ip in 1 ml). Under sterile conditions, 6-day-old ascites tumors were collected with a syringe, and the cells were separated from the ascitic fluid by low-speed centrifugation, washed in RPMI 1640 medium, resuspended (4×10^6 ml) in the same medium supplemented with 10% newborn calf serum, 1% (w/v) BSA (fraction V, fatty acid-free), and transferred to Petri dishes. Cells entered one of following treatments.

Treatment with clofibrate and mevalonate

Hepatoma cells were divided into four groups: *1*) control group, cells treated with solvent alone; *2*) clofibrate group, cells incubated with 0.75 mM concentration of clofibrate dissolved in dimethylsulphoxide; *3*) mevalonate group, incubated with 1 mM mevalonate, and mevalonic acid lactone was dissolved in 0.1 M NaOH and stirred for 2 h at 50°C; *4*) clofibrate plus mevalonate group, and cells incubated with both substances.

For each group, the hepatoma cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. At 0.5 h, 1 h, 2 h, and 4 h hepatoma cells were collected and centrifuged at 600 *g* for 10 min. Pellets and supernatants were used for all the following determinations performed.

Treatment with farnesyltransferase inhibitor

Hepatoma cells were divided into two groups: *1*) control group, cells treated with solvent alone; *2*) α-hydroxyfarnesylphosphonic acid (HFPA) group, cells incubated with 1 μM HFPA, farnesyltransferase inhibitor.

For each group, the hepatoma cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. At 2 h, 4 h, and 24 h hepatoma cells were collected and centrifuged at 600 *g* for 10 min. The pellets and supernatants were used for determination of apoptotic index and DNA distribution.

Treatment with simvastatin

Hepatoma cells were treated as at points 1 and 2 of first treatment except that simvastatin (0.5 mM dissolved in ethanol, maximum final concentration 0.25%) was added instead of clofibrate. The cells were used for apoptosis and cholesterol determinations.

Treatment with cyclosporin A

Hepatoma cells were treated as with the clofibrate and mevalonate group, except that 1 μM cyclosporin was added instead of mevalonate. Cyclosporin was added to the cells 15 min before clofibrate. The cells were used for apoptosis determination.

Semiquantitative RT-PCR of HMGR

mRNA content was measured by RT-PCR. Primers were designed by seeking suitable target sequences in the coding region of rat HMGR mRNA (AN: NM_013134). Two primers (Biotez Berlin-Buch GmbH, Berlin, Germany) generating a 684 base product were synthesized. The sequences of the forward and reverse primers were 5′-CATGAACATGTTCACCGGC-3′ and 5′-AGACATGATCATCTTGACCC-3′, respectively, and hybridized to the bases 99–117 and 764–783 of HMGR coding region. RT-PCR was performed by using the Access RT-PCR system (Promega Italia, Milan, Italy) following the manufacturer's instructions. Amplification profile was: 1 min 95°C, 1 min 52°C, 30 s 72°C for a

number of cycles ranging from 15 to 25, sufficient to maintain the amplification of the target sequence in the linear ranger. For each sample, amounts of total RNA, extracted from cells treated or not with clofibrate (see below), ranging from 10 ng to 150 ng, were-reverse transcribed and amplified. PCR products were resolved on a 2% ethidium bromide containing-agarose gel and the fluorescence generated by transillumination was acquired by using a Gel Doc System (BioRad, Milan, Italy) and analyzed with the software (Quantity One 4.0.3 BioRad, Milan, Italy). The ratio of HMGR versus β-actin fluorescence was then calculated for each sample and expressed by arbitrarily setting this ratio to 1 in control cells.

Northern blot analysis for PPAR and HMGR

Total RNA was prepared by the method of Chomczynski and Sacchi (29) and quantified by measuring the absorbance at 260 nm. Fifteen micrograms of RNA was heat-denaturated at 65°C for 10 min, applied to 1% agarose gel containing formaldehyde, electrophoresed, and transferred by capillary action to Hybond-N+ (Amersham, Buckinghamshire, UK). Prehybridization was in 50% formamide, 5× SSPE, 1× Denhardt's, 10% dextran, 1% SDS, and 100 μg/ml salmon sperm DNA at 42°C for 18 h. Hybridization conditions were identical to prehybridization. The DNA probes for PPAR α, PPARγ, and HMGR were labeled with [^{32}P]dCTP using a random primer DNA kit. Hybridization was carried out at 42°C for 24 h. Blots were washed once at room temperature in 2× SSC plus 0.5% SDS (15 min), once at 65°C in 2× SSC plus 0.5% SDS (15 min), once in 1× SSC plus 0.5% SDS (15 min), and once in 0.5× SSC plus 0.5% SDS (15 min). Kodak Hyperfilm-MP was exposed for 2 days with intensifying screens at −80°C.

HMGR activity

Microsomes were isolated from control and clofibrate-treated cells as described in Pascale et al. (30). Microsomal suspensions were immediately used to assay HMGR activity (31).

Lipid peroxidation

Lipid peroxidation was evaluated as malondialdehyde production by the spectrophotometric thiobarbituric acid assay (32).

DNA distribution

DNA staining was performed as described elsewhere (33). Briefly, cells were washed in PBS, fixed in ice-cold 70% ethanol for at least 30 min, and incubated at room temperature in PBS containing DNase-free RNase (Type II-A) and propidium iodide at final concentrations of 0.4 and 0.18 mg/ml, respectively. Cells were then analyzed with a FACScan flow cytometer (Becton and Dickinson, Mountain View, CA) equipped with a 488 nm argon laser and two filters, respectively transmitting at 585 nm (FL2) and above 620 nm (FL3). Data were recorded on a Hewlett Packard computer (HP 9000, model 300) using CellFit software (Becton and Dickinson).

Cell integrity

Plasma membrane integrity was checked microscopically. Trypan blue exclusion was evaluated on cells suspended in the presence of the dye (0.8 mg/ml). Four hundred cells were counted for each sample and the results expressed as percentages of trypan blue-positive cells.

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 1,000 cells per sample.

Isolation of mitochondria

Untreated cells, or those treated with 0.75 mM clofibrate or 0.5 mM simvastatin for 1 h and 2 h, were sedimented by centrifugation at 600 *g* for 10 min, homogenized by Potter-Helvehjem homogenizer in a volume of isolation medium A [70 mM sucrose, 220 mM mannitol, 20 mM Tris-HCl buffer, pH 7.4, 2 mM EGTA and 0.1% (w/v) albumin] corresponding to 0.5 × the mass of the pellet, and then diluted to 20% with the same medium. Mitochondria were isolated by Canuto et al. method (34). Isolated mitochondria were resuspended in isolation medium A (4 g/ml, w/v) and used for cholesterol content determination.

Cholesterol analysis

Total lipids were isolated by the Folch et al. method (35). Cholesterol and cholesterol ester contents were determined as described by Bowman and Wolf (36) on total cells and mitochondria.

Western blot analysis for p21 ras and cytochrome c

Cells treated or not with 0.75 mM clofibrate for 0.5 h, 1 h, or 2 h were sedimented by centrigugation at 600 *g* for 10 min, homogenized in a Potter-Helvejem homogenizer in a volume of isolation medium B [250 mM sucrose, 40 mM Tris-HCl buffer, pH 7.5; 10 mM $MgCl_2$, 2 mM $CaCl_2$, 1 mM PMSF, leupeptin (15 μg/ml)] corresponding to 5× the mass of the pellet, and then diluted to 10% with the same medium. Diluted homogenates were centrifuged at 105,000 *g* per 57 min. The pellets (total membranes) resuspended in medium B (2 g/ml, w/v) and the cytosol were used for Western blot analysis. Equal quantity of proteins of total membranes and cytosol were subjected to electrophoresis on 15% SDS-PAGE, transferred onto nitrocellulose membranes, and immunoblotted with monoclonal anti-Ras antibody. For cytochrome c, only cytosolic fractions were analyzed with a monoclonal anti-cytochrome c. The proteins were detected by a enanched chemiluminescent detection system.

Statistical analysis

Data are expressed as means ± SD. The significance of differences was assessed by ANOVA, followed by the Newman-Keuls test.

RESULTS

Clofibrate-induced apoptosis is not mediated by an increase of PPARα and PPARγ expression

Previous work from our laboratories has shown that addition of clofibrate to short-term cultures of AH-130 hepatoma cells rapidly induces the onset of apoptotic death (18, 19).

The action of PPs such as clofibrate usually relies on the activation of PPARs. However, the expression of mRNA for both PPARα and PPARγ, virtually absent in AH-130 hepatoma cells, is not induced by treatment with clofibrate, suggesting that PPARs are not relevant to the induction of cell death by clofibrate. **Figure 1** reports data relative to PPARα and PPARγ.

Since the failure to stimulate PPARα caused a failure of peroxisome proliferation as well as of oxygen free radical production, whose possible consequence is lipid peroxidation, the effect of clofibrate was also examined in terms of

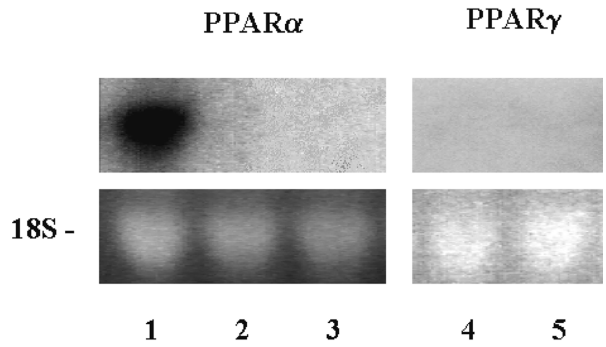

Fig. 1. Peroxisome proliferator activated receptor (PPAR)α expression in normal liver and AH-130 hepatoma cells and PPARγ expression in AH-130 hepatoma cells. Lane 1: normal liver; lane 2: control hepatoma cells; lane 3: hepatoma cells treated with 0.75 mM clofibrate for 2 h; lane 4: control hepatoma cells; lane 5: hepatoma cells treated with 0.75 mM clofibrate for 2 h.

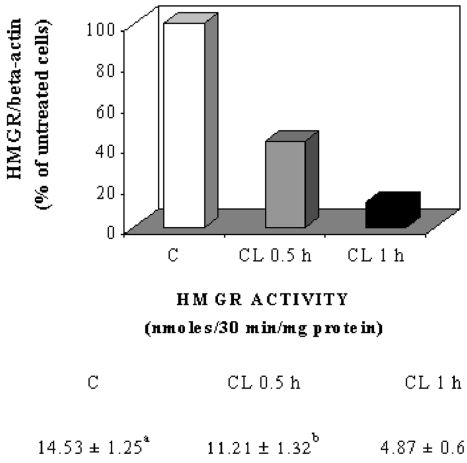

Fig. 2. HMG-CoA reductase (HMGR) mRNA level. HMGR expression and activity in AH-130 hepatoma cells treated with 0.75 mM clofibrate for 0.5 and 1 h. HMGR activity is expressed as nmoles of mevalonate produced for 30 min and for micrograms of protein. Data are means ± SD of three experiments. Means of groups with different letters are statistically different ($P < 0.05$) from one another as determined by variance analysis followed by the Newman-Keuls test. C: Control hepatoma cells: CL 0.5 h, hepatoma cells treated with clofibrate for 0.5 h; CL 1 h, hepatoma cells treated with clofibrate for 1 h.

lipid peroxidation. The colorimetric determination of malondialdehyde production, which is a good test to evaluate lipid peroxidation, showed no peroxidative breakdown of polyunsaturated fatty acids (data not shown).

Clofibrate-induced apoptosis is mediated by inhibition of HMGR

To gain an insight into the possible mechanisms underlying clofibrate-induced apoptosis, we focused our attention on this drug's interference with HMGR. HMGR controls the synthesis of cholesterol and isoprenoid units, both of which are involved in the regulation of cell proliferation. Isoprenoids, in particular, are necessary for the farnesylation of proteins that modulate several signaling transduction pathways, such as p21 ras, which is involved in cell proliferation, differentiation, apoptosis, cytoskeletal organization, and membrane trafficking (24). The interference with HMGR may thus have several implications relevant to the action of clofibrate on hepatoma cells.

In order to check whether HMGR plays a role in clofibrate-induced apoptosis, HMGR mRNA level and activity were determined in AH-130 hepatoma cells by RT-PCR and radioactivity assay respectively. **Figure 2** shows a reduction of HMGR mRNA level and activity in hepatoma cells exposed to clofibrate in a correlation that depends on clofibrate exposure time and apoptotic index (**Fig. 3**). These data seem to point to interference with HMGR as one of the possible mechanisms of clofibrate-induced apoptosis. To be sure that HMGR mRNA is effectively reduced, the content of HMGR mRNA was also evaluated by Northern blot analysis, confirming its reduction due to treatment with clofibrate (data not shown).

It is well known that the inhibition of HMGR can be overcome by supplying mevalonate, the downstream reaction intermediate. However, addition of mevalonate to AH-130 hepatoma cells treated with clofibrate partially prevents clofibrate-induced apoptosis (Fig. 3), as shown by staining the cells with DAPI. One millimole of meval-

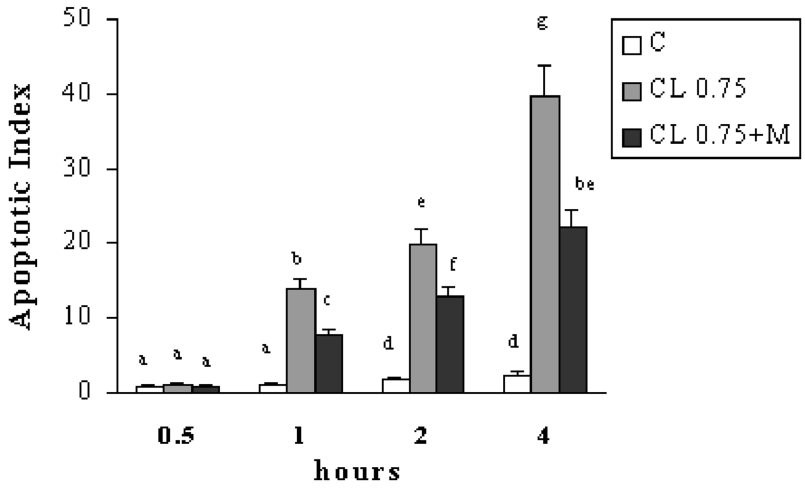

Fig. 3. Apoptotic index (percentage of apoptotic cells) in AH-130 hepatoma cells treated with clofibrate and mevalonate for 0.5 h, 1 h, 2 h, or 4 h. Cells stained with the DNA specific fluorochrome 4′,6′-diamidino-2′-phenylindole (DAPI) were examined under an epi-illuminated fluorescence microscope. At least 1,000 cells per sample were counted. Data are means ± SD of four experiments. Means of groups with different letters are statistically different ($P < 0.05$) from one another as determined by variance analysis followed by the Newman-Keuls test. C: Control hepatoma cells: CL 0.75, hepatoma cells treated with 0.75 mM clofibrate; CL 0.75 + M, hepatoma cells treated with 0.75 mM clofibrate and 1 mM mevalonate.

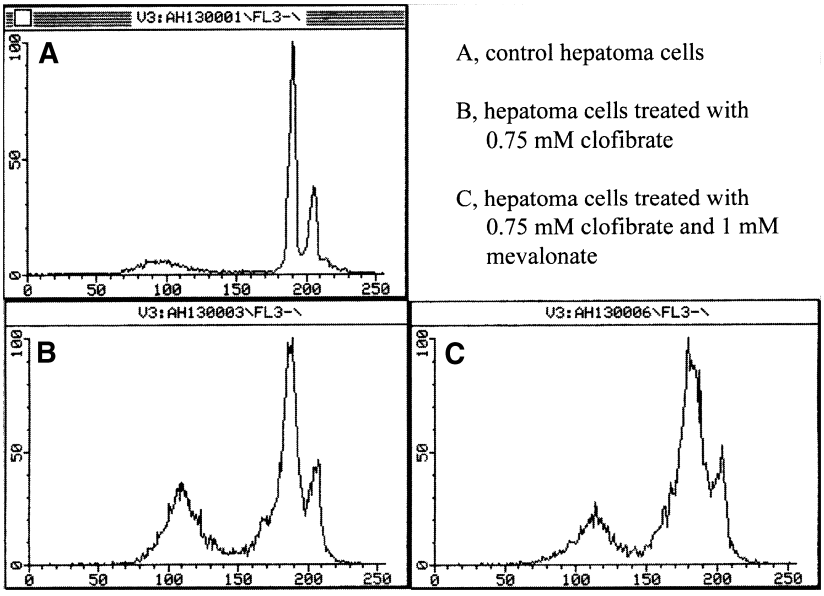

Fig. 4. DNA distribution detected by flow cytometry in AH-130 hepatoma cells. The cells were examined 2 h after treatment with 0.75 mM clofibrate and 1 mM mevalonate.

onate added to the cells at the same time as 0.75 mM clofibrate reduced the apoptotic index to about 50% at both 2 h and 4 h. These data were confirmed by flow cytometric analysis of DNA content. A significant proportion of clofibrate-treated cells has a hypodiploid DNA content, indicative of apoptotic figures. When AH-130 hepatoma cells were incubated with both clofibrate and mevalonate, the percentage of cells in the A_0 region (apoptotic cells) was significantly reduced (**Fig. 4**). To rule out the possibility of mevalonate itself being apoptogenic, hepatoma cells were treated with mevalonate alone. The results at the various experimental times confirmed that mevalonate alone had no effect in determining apoptosis (data not shown). Moreover, to exclude the possibility that mevalonate was necrogenic, we measured trypan blue exclusion by hepatoma cells. The percentage of unstained cells showed that mevalonate did not cause necrosis; over 95% of cells excluded trypan blue and were thus considered either viable or apoptotic (data not shown).

Cholesterol biosynthesis is relevant in the induction of apoptosis

The analysis of lipid content of AH-130 hepatoma cells treated with clofibrate showed that a significant decrease of cholesterol levels occurs at 1 h of treatment in mitochondria, whereas a significant decrease occurs only at 4 h of treatment in whole cells, when clofibrate cytotoxicity has fully developed (**Fig. 5**). **Figure 6** also shows that cholesterol esters decreased in a time-dependent manner in the presence of clofibrate. The same behavior occurred with simvastatin. To clarify whether the decrease of cholesterol in mitochondria was only due to clofibrate, or could also be caused by other hypolipidemic drugs, hepatoma cells were treated with simvastatin, which is a known HMGR inhibitor. Simvastatin also induced apoptosis in hepatoma cells at similar concentrations, as did clofibrate. At 0.5 mM, after 1 h and 2 h there were about 10% and 20% of apoptotic cells, respectively, whereas in the control, only 2% of cells were in apoptosis. Mitochondria were isolated from simvastatin-treated cells and analyzed for cholesterol and cholesterol ester contents. The results for simvastatin-treated cells, reported in Figs. 5 and 6, show a decrease of both cholesterol and cholesterol esters, as occurred in mitochondria obtained from cells treated with clofibrate.

Besides cholesterol biosynthesis, the relevance of HMGR in the induction of apoptosis by clofibrate might derive from the role played by this enzyme in production of isoprenoid units for farnesylation of proteins involved in several signaling transduction pathways as stated above (24). To evaluate whether this mechanism may be evoked in clofibrate-induced apoptosis, AH-130 hepatoma cells were examined for protein content of p21 ras, whose farnesylation is necessary for its activity, and treated with an inhibitor of farnesyltransferase, the α-hydroxyfarnesylphosphonic acid (HFPA). With regard to protein content of p21 ras, there were no variations in either membranes or cytosol isolated from hepatoma cells treated with clofibrate (**Fig. 7**). Moreover, no evidence of apoptosis was observed in HFPA-treated cells until 4 h of treatment, the percentages of cells in the A_0 region, corresponding to content of hypodiploid DNA, being 6.45% at 4 h and 13% at 24 h of treatment (**Fig. 8**).

Clofibrate-induced apoptosis is mitochondria dependent

The decrease of cholesterol in mitochondria may cause a perturbation in mitochondrial membrane potential, after which a release of cytochrome c from mitochondria to cytosol may occur. To verify this occurrence, the content of cytochrome c was evaluated by Western blot analysis in

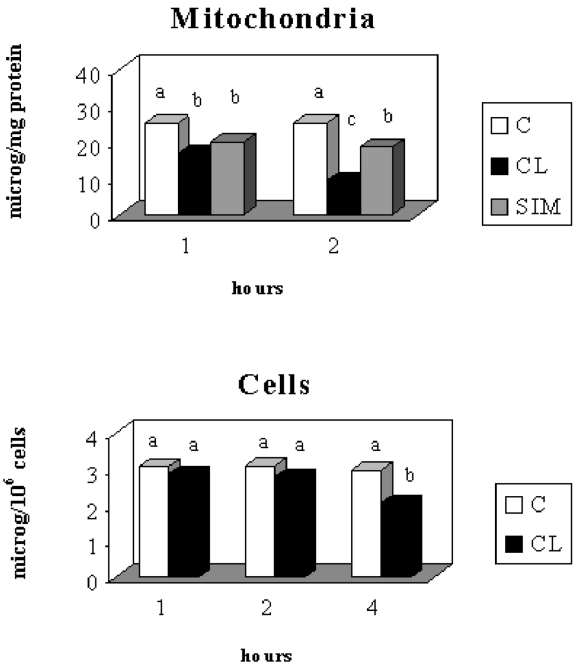

Fig. 5. Content of cholesterol in isolated mitochondria and in AH-130 hepatoma cells treated with 0.75 mM clofibrate or 0.5 mM simvastatin. The results are expressed in micrograms of free cholesterol/mg of protein for mitochondria and as micrograms of free cholesterol/10^6 cells for whole cells. They represent the means ± SD of three experiments. SD, not reported in the figure, is below 10%. For each panel, means of groups with different letters are statistically different ($P < 0.05$) from one another as determined by variance analysis followed by the Newman-Keuls test. C, control hepatoma cells; CL, hepatoma cells treated with 0.75 mM clofibrate; SIM, hepatoma cells treated with 0.5 mM simvastatin.

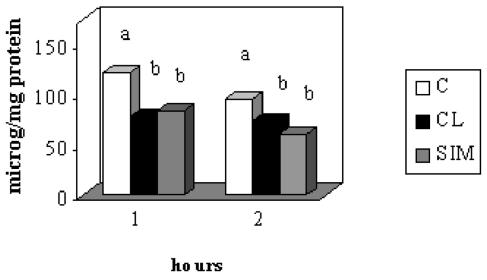

Fig. 6. Content of cholesterol esters in mitochondria isolated from AH-130 hepatoma cells treated with 0.75 mM clofibrate or 0.5 mM simvastatin. The results are expressed in micrograms of cholesterol esters/mg of protein. They represent the means ± SD of three experiments. SD, not reported in the figure, is below 10%. For each panel, means of groups with different letters are statistically different ($P < 0.05$) from one another as determined by variance analysis followed by the Newman-Keuls test. C, control hepatoma cells; CL, hepatoma cells treated with 0.75 mM clofibrate; SIM, hepatoma cells treated with 0.5 mM simvastatin.

cytosol isolated from cells treated or not with clofibrate, as shown in **Fig. 9**.

At 1 h cytochrome c was present in the cytosol and further increased at 2 h of treatment. Release of cytochrome c in the cytosol is one of the central events of apoptosis (25).

To demonstrate that, in cells treated with clofibrate, apoptosis is mitochondria-dependent, cells were pretreated with cyclosporin A, an inhibitor of mitochondrial permeability transition. Clofibrate-induced apoptosis was completely prevented. Apoptosis in cells treated with clofibrate was about 15% at 1 h of treatment, whereas in cells treated with clofibrate and cyclosporin the apoptosis rate was 2%.

DISCUSSION

Cell death by apoptosis induced in AH-130 hepatoma cells by clofibrate is characterised by shrinkage, membrane blebbing, chromatin condensation, and nuclear fragmentation, as demonstrated by morphological, flow cytometrical, and biochemical analyses (18, 19).

The observation that clofibrate may induce apoptosis seems to be in contrast with PP action, frequently believed to cause an altered balance between cell proliferation and death, favoring the former or stimulating cell growth or suppressing cell death by apoptosis (5, 26). By contrast, it has been shown that PPs can inhibit cell growth or induce apoptosis in some tumor cells (18–22).

The mechanisms by which clofibrate rapidly induces apoptosis are at the moment totally obscure. This drug may exert its effects by acting on PPARs, as well as by inhibiting the HMGR activity. The present study aimed to investigate whether the apoptogenic potential of clofibrate may depend on one of those pathways.

PPARs were discovered in 1990, ending 25 years of uncertainty about the molecular mechanisms responsible for peroxisome proliferation (37). PPARs are nuclear receptors and were initially described as molecular targets for compounds inducing peroxisome proliferation. They have since been found to be activated by a number of medically relevant compounds, such as the fibrate class of hypolipidemic drugs, thiazolidinediones (insulin sensitiz-

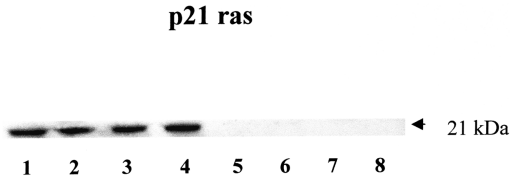

Fig. 7. Content of p21 ras protein in membranes and cytosol isolated from AH-130 hepatoma cells treated with 0.75 mM clofibrate for 1 h and 2 h. Lane 1: membranes isolated from control hepatoma cells treated with solvent alone for 1 h; lane 2: membranes isolated from hepatoma cells treated with clofibrate for 1 h; lane 3: membranes isolated from control hepatoma cells treated with solvent alone for 2 h; lane 4: membranes isolated from hepatoma cells treated with clofibrate for 2 h; lane 5: cytosol isolated from control hepatoma cells treated with solvent alone for 1 h; lane 6: cytosol isolated from hepatoma cells treated with clofibrate for 1 h; lane 7: cytosol isolated from control hepatoma cells treated with solvent alone for 2 h; lane 8: cytosol isolated from hepatoma cells treated with clofibrate for 2 h.

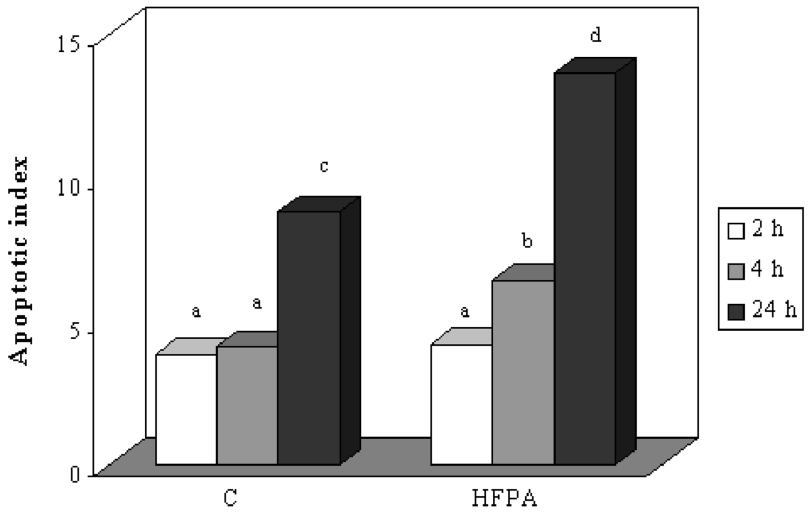

Fig. 8. DNA distribution detected by flow cytometry in AH-130 hepatoma cells. The cells were examined 2 h, 4 h, and 24 h after treatment with 1 μM α-hydroxyfarnesylphosphonic acid. Data, expressed as percentage of cells in the sub G0/G1 region over total cells counted, are means ± SD of three experiments. SD, not reported in the figure, are below 10%. Means of groups with different letters are statistically different ($P < 0.05$) from one another as determined by variance analysis followed by the Newman-Keuls test. C, control hepatoma cells; HFPA, hepatoma cells treated with 1 μM α-hydroxyfarnesylphosphonic acid.

ers used as oral antidiabetic agents), some non-steroidal anti-inflammatory drugs, and naturally occurring fatty acid-derived molecules (38).

We examined the expression of PPARα and PPARγ in clofibrate-treated AH-130 hepatoma cells and found that clofibrate did not increase the mRNA of either receptor. The induction of PPARα usually causes stimulation of peroxisome enzymes and, as a consequence, an increase of both oxygen free radical production and lipid peroxidation. Consistently with the lack of PPARα induction, AH-130 hepatoma cells exposed to clofibrate did not show any increased lipid peroxidation (data not shown). These observations confirm our previous impression that an involvement of PPARs in mediating the lethal effect of clofibrate in AH-130 hepatoma cells was very unlikely (19).

The other possibility was that clofibrate cytotoxicity could result from the interference of the drug with HMGR, with consequences on both cholesterol and isoprenoid unit biosynthesis. The involvement of the latter molecules in the induction of cell death is supported by some studies showing that the block of mevalonate synthesis exerted by lovastatin, another HMGR inhibitor, impairs isoprenylation of Ras and activation of the MAP kinase cascade (39), or geranylgeranylation of RhoA, affecting RhoA pathway (40) and resulting in down-regulation of cell proliferation. Consistently, lovastatin has been shown to suppress cell growth and to induce apoptosis in several types of tumor (41).

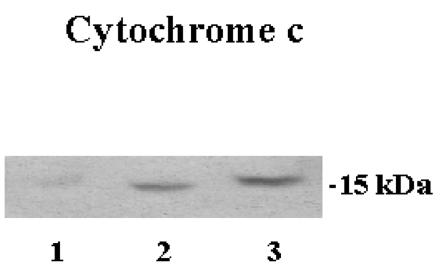

Fig. 9 Release of cytochrome c in the cytosol of AH-130 hepatoma cells treated with 0.75 mM clofibrate. Lane 1: control hepatoma cells; lane 2: hepatoma cells treated with clofibrate for 1 h; lane 3: hepatoma cells treated with clofibrate for 2 h.

The results reported here seem to point to HMGR and cholesterol as possible mediators of clofibrate-induced apoptosis. Indeed, clofibrate causes a marked reduction of HMGR mRNA content and activity, and the addition of mevalonate to clofibrate-treated AH-130 cells results in partial protection from cell death. To discover which of the two pathways depending on HMGR activity is affected by clofibrate, we examined both cholesterol content ad farnesylation pathway. From our results, the decrease of cholesterol content appears to be involved, whereas farnesylation does not appear to be important in inducing apoptosis at the earlier treatment times. Cholesterol is increased in mitochondria of AH-130 hepatoma cells in comparison with normal liver, as also occurs in Morris hepatoma and hepatoma chemically induced in rats as we have shown elsewhere (27, 28). The cholesterol increase in mitochondria causes changes in physical and functional properties (27), and could be involved in preventing apoptosis in hepatoma cells. Treatment with clofibrate decreases cholesterol and cholesterol esther content in mitochondria isolated from hepatoma cells, already at the earlier treatment times. Treatment with simvastatin also causes a decrease of both cholesterol and cholesterol esther content in mitochondria. Moreover, the cholesterol decrease closely correlates with the release of cytochrome c from mitochondria, which is an important step in activating caspase-mediated apoptosis (25). The fact that mitochondria are the major target of clofibrate cytotoxicity was confirmed by the observartion that cyclosporin A inhibits clofibrate-induced apoptosis and that caspase 9 and 3 are activated, whereas caspase 8 is only marginally increased (submitted for publication).

We found no involvement of alterations of protein farnesylation in clofibrate-induced cell death, at least at the early stages of treatment, since p21 ras localization (membranes and cytosol) did not vary between control and treated cells, and since apoptosis did not occur in AH-130 cells exposed to HFPA, an inhibitor of farnesyltransferase,

at the early stages of treatment. This observation suggests that inhibition of protein farnesylation requires more time to produce cell death, and rules out the possibility that such a mechanism may dominate in clofibrate-induced cells, especially during the first hours of treatment.

In conclusion, the bulk of our results show that the apoptogenic effect of clofibrate does not depend on induction of PPARs, but that the role played by HMGR reduction is relevant, and that the mechanism inducing apoptosis is through cholesterol decrease in mitochondria and cytocrome c release.

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