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Mechanisms of clofibrate-induced apoptosis in Yoshida AH-130 hepatoma cells

F. Penna, P. Reffo, G. Muzio, R.A. Canuto, F.M. Baccino, G. Bonelli, P. Costelli *

Department of Experimental Medicine and Oncology, University of Torino, Corso Raffaello, 30, 10125 Torino, Italy

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ABSTRACT

Peroxisome proliferators (PPs) are a class of compounds that exert their nominal effects through the peroxisome proliferator-activated receptors. PPs, among which clofibrate (CF), have been extensively studied for their hepatocarcinogenic properties in rodents, generally ascribed to their antiapoptotic action. However, previous results demonstrated that various PPs may also have apoptogenic properties. CF, in particular, promptly induces a massive apoptotic death in cell lines established from murine or human hepatomas and from breast or lung cancers as well.

The present study was aimed at elucidating the apoptotic pathway(s) triggered by CF in AH-130 cells. The results show that CF-induced cell death is completely blocked by the poly-caspase inhibitor z-VAD-fmk and that caspases 3, 8, and 9 are early activated. Consistently, cytochrome *c* is released from mitochondria, and CF cytotoxicity is inhibited by cyclosporine A, partially at least. In addition, the occurrence of endoplasmic reticulum (ER) stress is suggested by the observation that the levels of phosphorylated eIF2α and JNK increase in CF-treated cells, while the caspase 2 precursor protein levels are concurrently reduced. Finally, some degree of calpain activation also takes place, as suggested by the appearance of fodrin cleavage products.

The present findings demonstrate that CF-induced apoptosis in the Yoshida AH-130 cells basically is a caspase-dependent process that involves more than a single mechanisms. Activation of the intrinsic apoptotic pathway and ER stress both play a major and concurrent role, while calpain activation seems to have only a marginal part in the process.

1. Introduction

Peroxisome proliferators (PPs) are a class of heterogeneous compounds that include industrial chemicals, pharmaceuticals, and biomolecules such as fatty acids and eicosanoids. Their effects are mediated by the PP-activated receptors (PPARs). This subgroup of the nuclear hormone receptor superfamily is comprised of three molecular forms, namely PPARα, β/δ, and γ, that are differentially expressed in adult and embryonic tissues [1]. Upon ligand binding, PPARs dimerize with retinoid receptors, translocate to the nucleus, recognize specific PP-responsive elements (PPRE) on DNA and transactivate a number of genes [2,3]. PPARs have been involved in the regulation of processes such as intracellular lipid trafficking, mitochondrial and peroxisomal fatty acid uptake and β-oxidation, inflammation, cell proliferation and death. In addition, depending on the environment, they have been proposed to act as tumor suppressors or as tumor promoters.

PPs have been categorized as hepatocarcinogens in rodents [reviewed in 4]. Their administration to rats and mice indeed results in peroxisome proliferation, liver hypertrophy and hyperplasia, and hepatocarcinogenesis [5,6], while monkeys,

* *Corresponding author*. Tel.: +39 0116707062; fax: +39 0116707753.
E-mail address: paola.costelli@unito.it (P. Costelli).
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pigs and humans appear quite resistant to such effects [7–9]. PPs are usually regarded as non-genotoxic carcinogens, and their oncogenicity seems to depend on the oxidative stress caused by peroxisome proliferation as well as on their capability to alter the balance between cell proliferation and death [10,11]. This action is mediated through activation of PPARα, as shown by studies reporting that PPARα-null mice are refractory to hepatocarcinogenesis induced by long term PPs administration [12].

Among PPs, particular attention has been given to fibrate derivatives, which include agents that have been and still are largely used as hypolipidemic drugs in view of their ability to lower plasma triglyceride levels by accelerating mitochondrial fatty acid β-oxidation through PPARα activation [13]. Like other fibric acid derivatives, clofibrate (CF) has been widely employed in hepatocarcinogenesis protocols for rodents [14,15], in which its antiapoptotic action is assumed to play an important role. However, previous results from our laboratory have demonstrated that treatment with CF promptly induces a massive and typical apoptosis in hepatoma cells of both murine (Yoshida AH-130) and human origin (HepG2) [16]. Since then these observations have been extended to other PPs. Indeed, nafenopin is able to induce apoptotic death in the AH-130 cells [16], while perfluorooctanoic acid and BR931 exert cytotoxic effects on the HepG2 hepatoma cell line [17,18]. In addition, PPARα activation has been shown to enhance hepatocyte apoptosis [19]. PPs cytotoxicity is not restricted to cells of hepatic origin, since similar effects have been observed in breast or lung cancer cell lines [20,21] as well as in human keratinocytes and lymphoblasts [22,23]. Besides PPARα, also PPARγ can induce cell death. Its activation, both in vitro and in vivo, triggers apoptosis through a MAPK-dependent mechanism [24,25], while in lung cancer cells it potentiates the antitumor effect of MK886, an inhibitor of arachidonic acid metabolism [26].

The mechanisms involved in CF-induced apoptosis are far from being elucidated. Previous results obtained in our laboratory suggested that a role may be played by inhibition of HMG-CoA reductase (HMGR), a key enzyme in isoprenoid biosynthesis. Indeed, the mRNA level and enzymatic activity of HGMR as well as the cholesterol content in mitochondria are reduced in Yoshida AH-130 cells soon after CF treatment, while cell death can be attenuated by supplementing cells with mevalonate, the reaction product of HMGR [27]. The present study, aimed at clarifying the nature of the apoptotic pathway(s) activated by CF in AH-130 cells, indicates that multiple processes are triggered by this agent, including (i) activation of the intrinsic pathway, with release of cytochrome *c* from mitochondria and activation of caspases 8, 9, and 3, (ii) endoplasmic reticulum (ER) stress as manifested by phosphorylation of eIF2α and JNK, associated with caspase 2 activation, and (iii) some degree of calpain activation, as inferred from the fodrin cleavage pattern.

2. Materials and methods

All materials were purchased from Sigma (St. Louis, MO, USA), unless specified differently.

The Yoshida AH-130 ascites hepatoma was maintained by weekly i.p. transplantation in male Wistar rats (20–30 × 10^6 cells/rat, Charles River, Como, Italy). Six-day-old tumors were collected under sterile conditions, 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% fetal calf serum and transferred to Petri dishes. CF, dissolved in DMSO, was added to the medium at the final concentration of 1 mM. Controls were treated with the solvent alone. In some experiments AH-130 cells have been pre-treated with one of the following inhibitors: z-VAD-fmk (1 h, 20, 50 or 100 μM), DEVD-cho (1 h, 100 μM), LEHD-cho (1 h, 100 μM), IETD-cho (1 h, 100 μM) from Alexis Biochemicals (Lausen, Switzerland), cyclosporin A (15 min, 1 μM), calpeptin (1 h, 250 μM) or BAPTA-AM (1 h, 10 μM) from Biomol (Plymouth Meeting, PA, USA).

2.1. *Flow cytometry*

DNA distribution analysis has been performed as described elsewhere [28]. Briefly, cells were washed in PBS, fixed in ice-cold 70% ethanol for at least 30 min, incubated at room temperature in PBS containing DNase-free RNase (Type II-A) and propidium iodide at the final concentrations of 0.4 mg/ml and 10 μg/ml, respectively. The cells were then analyzed with a FACScan flow cytometer (Becton & Dickinson, Mountain View, CA, USA) equipped with a 488 nm argon laser and three filters, respectively, transmitting at 530 nm (FL1), 585 nm (FL2) and above 620 nm (FL3). Data were analysed with the CellQuest software (Becton & Dickinson). The percentage of apoptotic events has been assessed by evaluating the accumulation of cells characterized by a $<2n$ DNA fluorescence [cf. 28].

Mitochondrial depolarization has been detected on unfixed cells measuring the fluorescence emission shift of the lipophilic cationic probe 5,5-6,6-tetra-chloro-1,1-3,3-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1, Molecular Probes, Invitrogen Corporation, Carlsbad, CA, USA). JC-1 exhibits potential-dependent accumulation in mitochondria, indicated by a fluorescence emission shift from green (529 nm) to red (590 nm). The loss of mitochondrial membrane potential is indicated by a decrease of the ratio between red and green fluorescence [29].

2.2. *Caspase activities*

Cells were resuspended in 20 mM HEPES-KOH, pH 7.5, containing 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, frozen and thawed, sonicated, centrifuged (14,000 × *g*, 15 min, 4 °C), and the supernatant collected. Aliquots corresponding to 20 μg protein were diluted in caspase buffer (25 mM HEPES, pH 7.5, 0.1% CHAPS, 10% sucrose, 10 mM DTT) and assayed for caspase 3, 8, and 9 activities by 1 h incubation at 37 °C in the presence of 20 μM substrates, respectively, DEVD-AMC, IETD-AMC, LEHD-AMC (Biomol Plymouth Meeting, PA, USA). The reaction was stopped with 0.1% TCA, and the fluorescence read in a PerkinElmer fluorometer (excitation 380 nm – emission 460 nm). Free AMC was used as working standard.

2.3. Western blotting

Cells were suspended in 0.25% sodium deoxycholate and homogenized by sonication. For cytochrome c detection, cells were suspended in 20 mM HEPES-KOH, pH 7.2, containing 250 mM sucrose, 1 mM EDTA, 0.025% digitonin and freshly added 0.1 mM PMSF, and centrifuged (14,000 × g, 30 min, 4 °C) to obtain the mitochondrial (pellet) and the cytosolic (supernatant) fractions.

Protein concentration was determined by the method of Lowry, using BSA as working standard. Equal amounts of protein (50 μg) were heat-denaturated in sample-loading buffer (50 mM Tris–HCl, pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol), resolved by SDS-PAGE and transferred for 2 h to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). Protein transfer was checked by Ponceau S staining. The filters were then blocked with Tris buffered saline (TBS) containing 0.05% Tween and 5% non-fat dry milk and incubated overnight with the following primary antibodies: poly-ADP-ribose polymerase (PARP), caspase 2, caspase 12, fodrin, JNK (Santa Cruz Biotechnology, Santa Cruz, CA, USA); p-eIF2α, eIF2α, p-JNK (Cell Signaling, Danvers, MA, USA); cytochrome c (Becton & Dickinson, Mountain View, CA, USA). Peroxidase-conjugated IgG (Bio-Rad, Hercules, CA, USA) was used as secondary antibody. The membrane-bound immune complexes were detected by an enhanced chemiluminescence system (Santa Cruz Biotechnology, Santa Cruz, CA, USA) on a photon-sensitive film (Hyperfilm ECL; GE Healthcare, Milano, Italy). Bands were quantified by densitometric scanning of the films and elaborated as described in 'Data analysis and presentation'. The filters were then stripped by incubation in 62.5 mM Tris–HCl, pH 6.7, containing 100 mM 2-mercaptoethanol and 2% SDS for 30 min at 50° C, and reprobed with a mouse polyclonal antibody directed against tubulin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) to normalize sample loading.

2.4. Data analysis and presentation

Each experiment has been conducted in triplicate and repeated three times. Quantification was performed by densitometric analysis: bands were analyzed using a specific software (TotalLab, NonLinear Dynamics, Newcastle upon Tyne, UK). All results shown are expressed as mean ± SD. Significance of the differences has been evaluated by the Student's *t*-test.

3. Results

Previous studies performed in our laboratory demonstrated that treatment of Yoshida AH-130 cells with 0.5–1 mM CF results in rapid and extensive cytotoxicity [16]. The apoptotic nature of this cell death was established by morphological, biochemical and flow cytometric criteria. Indeed, cell shrinkage due to nuclear and cytoplasmic condensation is clearly detectable early in the course of CF treatment, associated with the appearance of a cell population with a $<2n$ DNA content and a corresponding degree of internucleosomal DNA degradation [16].

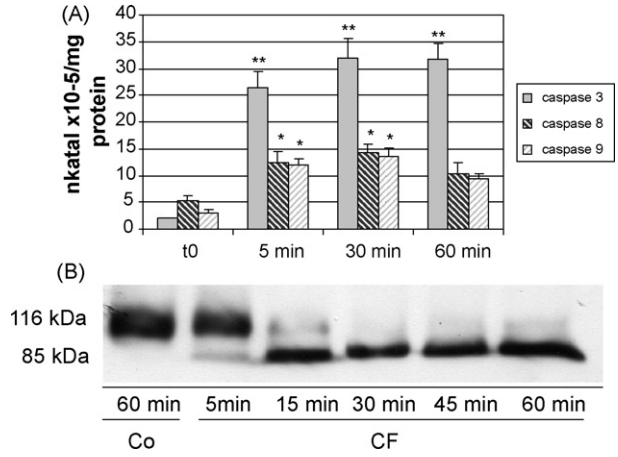

Fig. 1 – Caspase activation in clofibrate-treated AH-130 cells. (A) Caspase enzymatic activity evaluated by measuring the cleavage of specific fluorogenic substrates. Data (means ± SD) are expressed as nkatal × 10^{-5}/mg protein. Significance of the differences vs t_0: $^{*}p < 0.05$, $^{**}p < 0.01$ and (B) Western blot representative of the appearance of the 85 kDa PARP fragment during CF treatment.

To characterize the apoptotic pathway(s) triggered by CF in the AH-130 cells, the enzymatic activities of both initiator (8 and 9) and effector (3) caspases have been assayed by determining the cleavage of selective fluorogenic substrates. As shown in Fig. 1A, these caspase activities are all markedly increased already after a 5 min incubation of AH-130 cells with CF. Caspase 3 activity reaches a maximum at 30 min and maintains this level till the end of the experiment, while the activity of both caspases 8 and 9 slightly declines at 60 min. The occurrence of caspase 3 activation in vivo is confirmed by the early and extensive PARP cleavage (Fig. 1B).

Caspase activation is crucial to the onset of apoptotic death, since pretreating AH-130 cells with various caspase inhibitors affords a significant protection against CF. In particular, the poly-caspase inhibitor z-VAD-fmk almost completely prevents apoptosis and a strong protection is also afforded by DEVD-cho (caspase 3 inhibitor), while IETD-fmk and LEHD-fmk, respective inhibitors of caspases 8 and 9, show a weaker effect (Fig. 2).

The activation of caspase 9 suggests that CF-induced death of AH-130 cells involves the mitochondrial apoptotic pathway. In fact, as indicated by the increased green to red fluorescence ratio in the flow-cytometric JC-1 test, a clearcut collapse of mitochondrial membrane potential is detectable in AH-130 cells as soon as after 5 min incubation with the drug (Fig. 3A). This change is associated with release of cytochrome c to the cytosolic fraction (Fig. 3B). To verify how critical is the role played by mitochondria in CF-induced apoptosis, the AH-130 cells have been pretreated with cyclosporin A, a drug that can prevent the opening of the permeability transition pore, thus inhibiting both mitochondria depolarization and cytochrome c release. The results in Fig. 4 indicate that the mitochondrial pathway accounts only partially for CF-induced apoptosis, suggesting that additional mechanisms are involved as well.

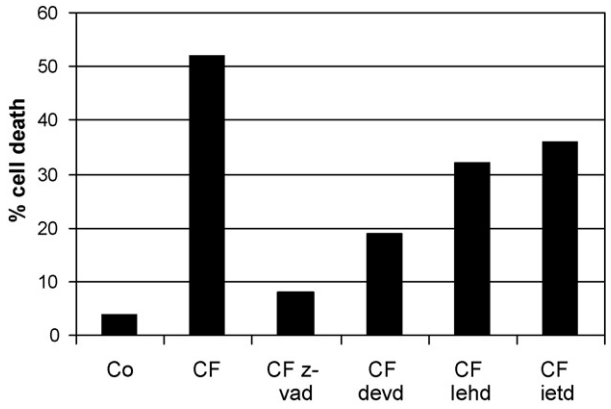

Fig. 2 – Effect of different caspase inhibitors on apoptotic death in AH-130 hepatoma cells exposed to clofibrate. Cells were pretreated with the different inhibitors (z-VAD-fmk: poly-caspase; DEVD-cho: caspase 3, IETD-fmk: caspase 8, LHED-fmk: caspase 9. Concentrations used: 100 μM, for 1 h), exposed to CF and analysed after 1 h. Histograms refer to precentages of cells with hypodiploid DNA content (apoptotic; see ref. [16]).

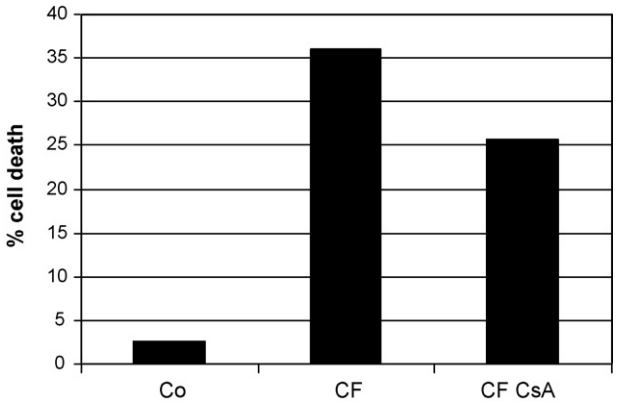

Fig. 4 – Effect of cyclosporine A (CsA) on cell death in AH-130 hepatoma cells exposed to clofibrate. Cells were pretreated with CsA (1 μM, 15 min), then exposed to CF and analysed after 1 h. Histograms refer to precentages of cells with hypodiploid DNA content (apoptotic; see ref. [16]).

Extrinsic and intrinsic apoptotic pathways apart, cell death may also result from the induction of endoplasmic reticulum stress [30]. Two typical responses in such a situation are the activating phosphorylation of SAPK/JNK and the inactivating phosphorylation of eIF2α, the latter resulting in inhibition of protein synthesis. In the AH-130 cells, a 5 min exposure to CF is sufficient to increase markedly the degree of eIF2α phosphorylation, which reaches a maximum at 15 min and subsequently declines, though remaining significantly above control levels even at 60 min (Fig. 5A). With a similar kinetics, the degree of phosphorylation of JNK also increases in CF-treated cells (Fig. 5B). These data strongly suggest that CF-treated AH-130 cells develop an ER stress.

Caspase 12, together with caspases 2 and 4, has been causally involved in triggering apoptosis in situations of ER stress. Caspase 2 is early activated in response to CF, since its precursor form rapidly disappears in treated AH-130 cells (Fig. 6). By contrast, the caspase 12 precursor is maintained, ruling out an activation of this caspase (Fig. 6).

The mobilization of Ca^{2+} from the ER stores is a well known concomitant of ER stress and cytosolic Ca^{+2} elevations may lead to activation of the Ca^{2+}-dependent proteases calpains [31]. To investigate if CF-induced apoptosis involves calpain, different approaches have been used. The occurrence of calpain activation in vivo can be detected by estimating the cleavage of specific physiological substrates such as fodrin,

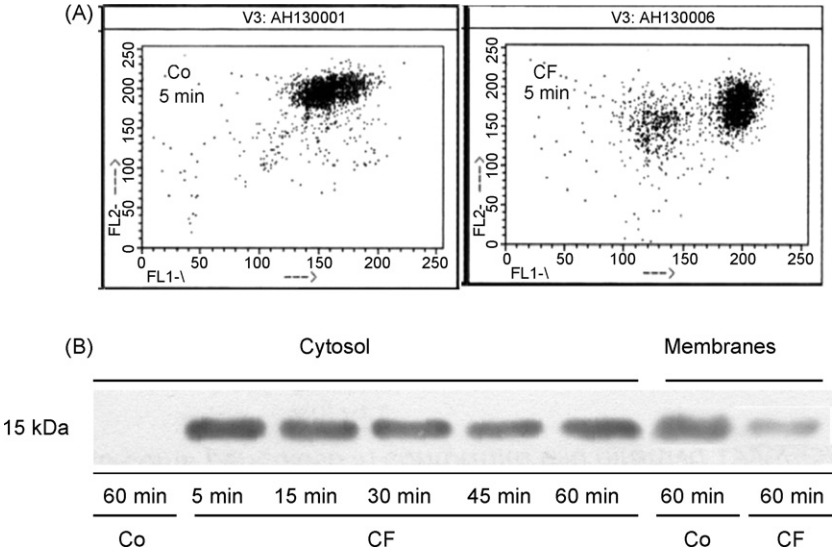

Fig. 3 – Mitochondrial depolarization and cytochrome c release in clofibrate-treated AH-130 cells. (A) Shift of the 590 nm/529 nm fluorescence ratio of JC-1 in cells treated with CF for 5 min and (B) representative western blot showing the time course of cytochrome c appearance in the cytosolic fraction (see Section 2 for details).

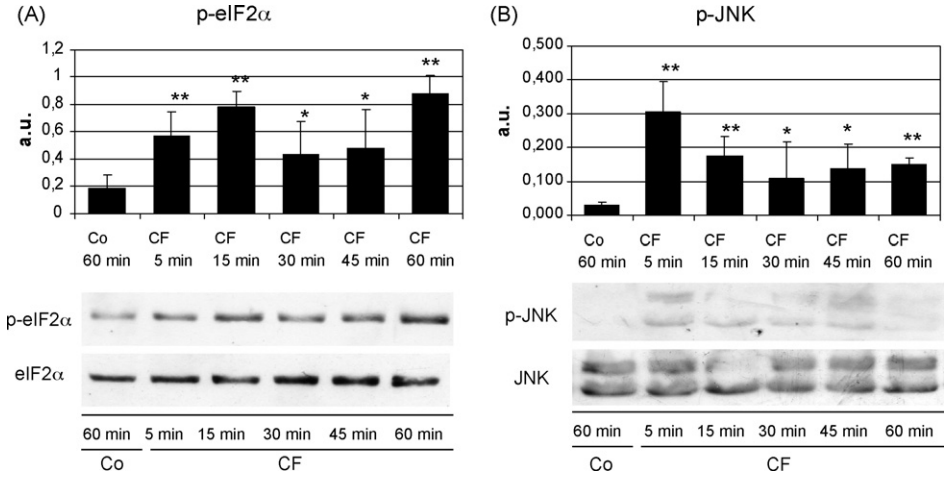

Fig. 5 – Levels of phosphorylated eIF2α and JNK in clofibrate-treated AH-130 hepatoma cells. Representative western blottings of (A) phosphorylated eIF2α and (B) phosphorylated JNK. Histograms refer to the densitometric analysis. Data (means ± SD) are expressed as arbitrary units. Significance of the differences vs controls (Co): $^{*}p < 0.05$, $^{**}p < 0.01$.

the 130 kDa Ca^{2+}-ATPase, and calpastatin [32]. Fodrin, in particular, can be cleaved by both calpain and caspase 3 to slightly different fragments of 150 kDa. In addition, calpain and caspase 3 also release a fragment of 145 or 120 kDa, respectively [reviewed in 32]. In CF-treated AH-130 cells the 150 kDa fragment is detectable by western blotting as soon as after a 5 min incubation, while the 120 kDa form becomes detectable after 45 min (Fig. 7A). The appearance of fodrin cleavage products can be totally abrogated by pretreating cells with the poly-caspase inhibitor z-VAD-fmk at a 100 μM concentration. However, the inhibition progressively decreases with z-VAD-fmk at 50 and 20 μM concentrations (Fig. 7B). This observation likely reflects a gradual loss of specificity of this inhibitor when used at increasing concentrations and, in particular, it involves caspase 3 in addition to calpain. In order to confirm the calpain activation, the AH-130 cells have been treated with CF in the presence of calpeptin, an inhibitor of calpain, or of BAPTA-AM, an intracellular Ca^{2+} chelator. The data presented in Fig. 8 show that while calpeptin partially abrogates CF-induced apoptosis, BAPTA-AM has no appreciable effect.

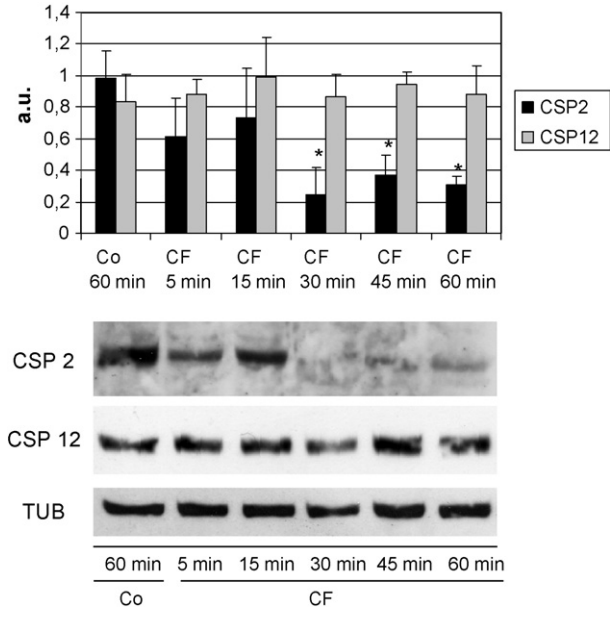

Fig. 6 – Time-course of caspase 2 and 12 activation in AH-130 cells exposed to clofibrate. Representative western blottings of caspase 2 and 12 activation. Histograms refer to the densitometric analysis. Data (means ± SD) are expressed as arbitrary units. Significance of the differences vs controls (Co): $^{*}p < 0.05$.

4. Discussion

Years ago we made the observation that PPs, CF in particular, can induce massive apoptosis of rat hepatoma cells. Although quite surprising at that time, in view of PPs carcinogenic and prosurvival properties, this report has by now gained a wide consensus since several studies have subsequently confirmed that various agents of this class can induce death in different tumoral and non-neoplastic cells as well as in the normal liver [16,18, 19, our unpublished observations]. As yet, little is known about the mechanisms underlying this cytotoxic action. CF is apoptogenic at 0.5–1 mM concentration, which apparently is quite a high one. It should be noted, however, that similar or even higher concentrations have been used in biochemical studies on tissue cultures [33]. More pertinently, in pharmacological terms, optimal therapeutic plasma levels of CF have been reported to be somewhere around 0.5 mM [34,35]. Moreover, 200–300 mg/kg b.w. per day is the usual CF dose in rat chemical hepatocarcinogenesis protocols [4]. We have previously reported that, at least in CF-induced AH-130 cell death, PPARα does not seem to be involved, which is consistent with the rapidity of the apoptotic response [27]. In spite of the well known oxidative properties of PP, lipid peroxidation is not increased in clofibrate-treated AH-130 cells [36] and, consistently, cell cannot be rescued from death by

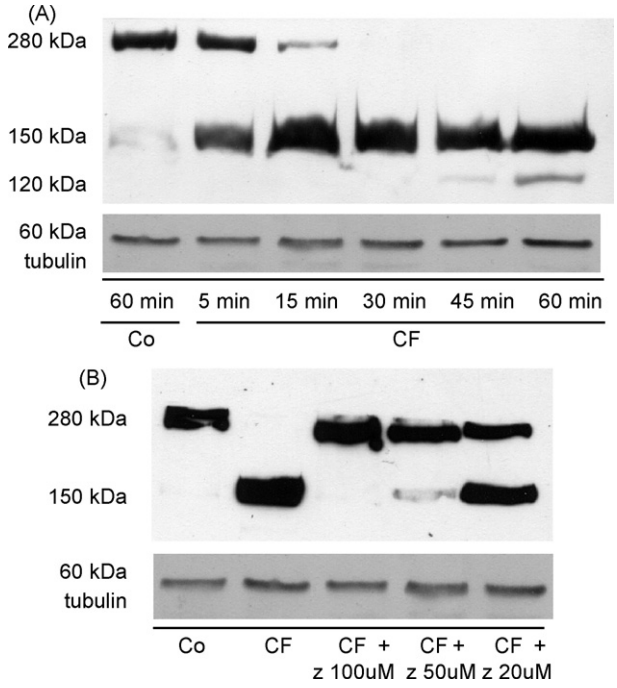

Fig. 7 – Fodrin cleavage in clofibrate-treated AH-130 cells. Representative western blots of (A) time-course of fodrin cleavage in CF-exposed AH-130 cells and (B) fodrin cleavage in the presence or in the absence of z-VAD-fmk. Cells were pretreated (1 h) with different concentrations of z-VAD-fmk (100, 50, 20 μM), then exposed to CF for 1 h.

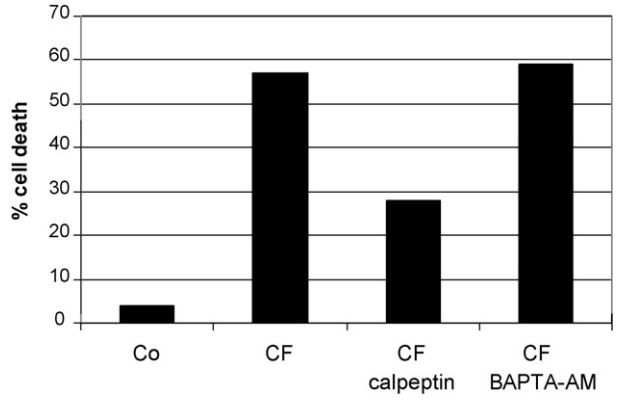

Fig. 8 – Effect of calpain inhibition on apoptotic death in AH-130 hepatoma cells exposed to clofibrate. Cells were pretreated with calpeptin (250 μM, 1 h) or with BAPTA-AM (10 μM, 1 h), then exposed to CF and analysed after 1 h. Histograms refer to precentages of cells with hypodiploid DNA content (apoptotic; see ref. [16]).

exposure to antioxidants such as BHT or N-acetyl-cysteine (percentage of apoptotic cells: CF = 45%, CF + BHT = 39%, CF + NAC = 53%). By contrast, CF toxicity can be attenuated by preloading cells with mevalonate [27]. This protection is far from complete, however, strongly suggesting that the contribution of reduced HMGR activity to CF cytotoxicity is only partial. The present study has thus been aimed to unravel the major mechanism(s) involved in CF-induced apoptosis.

Previous studies on L6 rat skeletal myoblasts and IM-9 human lymphoblasts reported that caspases are activated after 2–24 h of CF exposure [23,37]. In this regard, we found that in AH-130 cells caspases 3, 8 and 9 are activated as early as after 5 min of treatment with CF. Moreover, pretreatment of these cells with different caspase inhibitors affords a significant protection against apoptosis that is only partial with inhibitors that preferentially act on caspases 8, or 9, but extensive with an inhibitor of caspase 3 and virtually complete with the poly-caspase inhibitor z-VAD-fmk.

The involvement of caspase 9 suggests that CF may induce apoptosis by activating the intrinsic pathway. Our previous observations already showed that treatment of the AH-130 cells with CF causes the release of cytochrome *c* into the cytosol [27]. The present study further demonstrates that mitochondria depolarization associated with cytochrome *c* release occur very early (5 min) during incubation with CF. In addition, cyclosporin A, an inhibitor of the permeability transition pore, partially protects the cells from CF, to an extent comparable to that exerted by caspase 9 blockade with IETD-fmk. The mechanisms by which CF acts on mitochondria remain to be clarified, however. In this regard, cytochrome *c* release seems to depend on signals that can be delivered only in intact cells, since it does not occur when isolated mitochondria or whole AH-130 cell homogenates are directly incubated with CF (data not shown). Consistently, clofibrate incubation of the AH-130 cell homogenate does not result in increased caspase-3 activity ($C = 3.53 \pm 1.02$, $CF = 4.29 \pm 1.99$ nkatal $\times 10^{-5}$/mg protein).

CF has been reported to inhibit the ER to Golgi transport of newly synthesized proteins from the ER to the Golgi and to cause the retrograde movement of Golgi constituents back to the ER [38]. These findings suggest that CF may induce ER stress, a process that in the last years has been proposed as the trigger of novel cell death pathways. Both initiator (2, 8, 9) and effector (3, 4, 7) caspases have been involved [39], and caspase 12 has been proposed as a specific mediator of ER stress-induced apoptosis in rodent cells [40]. Recruitment of TNF receptor-associated factor 2 (TRAF2) and JNK phosphorylation have been observed in ER-induced cell death, resulting in activation of the apoptosis-signal-regulating kinase (ASK1) [41]. In addition, apoptosis by ER stress can be also regulated by proteins of the Bcl2 family [42] that, besides mitochondria and nucleus, can also localize to the ER and contribute to the regulation of the intracellular Ca^{2+} homeostasis [43]. Finally, ER stress is associated with inhibition of protein synthesis, and the enhanced phosphorylation of eIF2α is a reliable marker of such condition [44]. In the present study we report that CF activates an ER stress response in the AH-130 cells as shown by the early and marked increase in eIF2α and JNK phosphorylation. ER stress, however, does not result in caspase 12 recruitment, since the levels of procaspase 12 do not change during the whole experimental time. By contrast, a marked activation of caspase 2 occurs soon after exposure to CF, which is consistent with the complete protective effect exerted by the poly-caspase inhibitor z-VAD-fmk.

Ca^{2+} mobilization induced by ER stress or mitochondrial depolarization may activate the calpains, a class of Ca^{2+}-

dependent proteases, normally maintained in an inactive state in the cytosolic compartment. A number of proteins, among which protein kinase C, Cdk5, Ca^{2+}/calmodulin-dependent protein kinase IV, calcineurin, titin, calpastatin and fodrin have been proposed as in vivo calpain substrates [45]. In the last years, calpains have been also associated with procaspase 12 processing and activation [46]. More recently, L6 murine skeletal myocytes exposed to CF have been shown to undergo apoptotic death due to Ca^{2+}-dependent caspase 12 activation [37]. The results obtained in the present study, however, cannot clarify if calpains are activated in the AH-130 cells exposed to CF. In this regard, fodrin is cleaved to generate two fragments of 150 and 120 kDa, respectively. While the latter is characteristically produced by caspase 3, the former may also derive from calpain activity [32]. Both fragments disappear when the cells are pre-treated with 100 μM z-VAD-fmk, while only a weak reduction is obtained at a lower concentration (20 μM). This finding suggests that at the highest concentration the inhibitor that may also interfere with the activity of proteases other than caspases, such as calpains [47]. The involvement of calpain is also suggested by the partially protective effect exerted by calpeptin on CF-induced cell death. In spite of these results, however, the occurrence of calpain activation is questioned by both the lack of calpastatin cleavage (not shown) and by the observation that the Ca^{2+} chelator BAPTA-AM is unable to rescue the AH-130 cell from apoptosis.

In conclusion, the results reported in the present study demonstrate that CF-induced apoptosis of the Yoshida AH-130 cells is a caspase-dependent process that involves more than a single mechanisms. Particularly relevant in this regard are the activation of the intrinsic apoptotic pathway and the occurrence of ER stress, while even calpain may take some apparently marginal part in the process.

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REFERENCES

[1] Braissant O, Foufelle F, Scotto C, Dauça M, Wahli W. Differential expression of peroxisome proliferator-activated receptors (PPARs): tissue distribution of PPAR-alpha, -beta, and -gamma in the adult rat. Endocrinology 1996;137:354–66.
[2] Bardot O, Clemencet MC, Passilly P, Latruffe N. The analysis of modified peroxisome proliferator responsive elements of the peroxisomal bifunctional enzyme in transfected HepG2 cells reveals two regulatory motifs. FEBS Lett 1995;360:183–6.
[3] Park EY, Cho IJ, Kim SG. Transactivation of the PPAR-responsive enhancer module in chemopreventive glutathione S-transferase gene by the peroxisome proliferator-activated receptor-gamma and retinoid X receptor heterodimer. Cancer Res 2004;64:3701–13.
[4] Lai DY. Rodent carcinogenicity of peroxisome proliferators and issues on human relevance. J Environ Sci Health C Environ Carcinog Ecotoxicol Rev 2004;22:37–55.
[5] Reddy JK, Rao S, Moody DE. Hepatocellular carcinomas in acatalasemic mice treated with nafenopin, a hypolipidemic peroxisome proliferator. Cancer Res 1976;36:1211–7.
[6] Ohmura T, Ledda-Columbano GM, Piga R, Columbano A, Glemba J, Katyal SL, et al. Hepatocyte proliferation induced by a single dose of a peroxisome proliferator. Am J Pathol 1996;148:815–24.
[7] Lake BG, Rumsby PC, Price RJ, Cunninghame ME. Species differences in hepatic peroxisome proliferation, cell replication and transforming growth factor-beta1 gene expression in the rat, Syrian hamster and guinea pig. Mutat Res 2000;448:213–25.
[8] Bentley P, Calder I, Elcombe C, Grasso P, Stringer D, Wiegand HJ. Hepatic peroxisome proliferation in rodents and its significance for humans. Food Chem Toxicol 1993;31:857–907.
[9] Luci S, Giemsa B, Hause G, Kluge H, Eder K. Clofibrate treatment in pigs: effects on parameters critical with respect to peroxisome proliferator-induced hepatocarcinogenesis in rodents. BMC Pharmacol 2007;7:6.
[10] Peters JM, Cheung C, Gonzalez FJ. Peroxisome proliferator-activated receptor-alpha and liver cancer: where do we stand? J Mol Med 2005;83:774–85.
[11] Nishimura J, Dewa Y, Muguruma M, Kuroiwa Y, Yasuno H, Shima T, et al. Effect of fenofibrate on oxidative DNA damage and on gene expression related to cell proliferation and apoptosis in rats. Toxicol Sci 2007;97:44–54.
[12] Peters JM, Cattley RC, Gonzalez FJ. Role of PPAR alpha in the mechanism of action of the nongenotoxic carcinogen and peroxisome proliferator Wy-14,643. Carcinogenesis 1997;18:2029–33.
[13] Kliewer SA, Xu HE, Lambert MH, Willson TM. Peroxisome proliferator-activated receptors: from genes to physiology. Recent Prog Horm Res 2001;56:239–63.
[14] Reddy JK, Reddy MK, Usman MI, Lalwani ND, Rao MS. Comparison of hepatic peroxisome proliferative effect and its implication for hepatocarcinogenicity of phthalate esters, di(2-ethylhexyl) phthalate, and di(2-ethylhexyl) adipate with a hypolipidemic drug. Environ Health Perspect 1986;65:317–27.
[15] Cattley RC, Kato M, Popp JA, Teets VJ, Voss KS. Initiator-specific promotion of hepatocarcinogenesis by WY-14,643 and clofibrate. Carcinogenesis 1994;15:1763–6.
[16] Canuto RA, Muzio G, Maggiora M, Autelli R, Barbiero G, Costelli P, et al. Rapid and extensive lethal action of clofibrate on hepatoma cells in vitro. Cell Death Differ 1997;4:224–32.
[17] Shabalina IG, Panaretakis T, Bergstrand A, DePierre JW. Effects of the rodent peroxisome proliferator and hepatocarcinogen, perfluorooctanoic acid, on apoptosis in human hepatoma HepG2 cells. Carcinogenesis 1999;20:2237–46.
[18] Simbula G, Pibiri M, Sanna L, Cossu C, Molotzu F, Columbano A, et al. The peroxisome proliferator BR931 kills FaO cells by p53-dependent apoptosis. Life Sci 2004;75:271–86.
[19] Xiao S, Anderson SP, Swanson C, Bahnemann R, Voss KA, Stauber AJ, et al. Activation of peroxisome proliferator-activated receptor alpha enhances apoptosis in the mouse liver. Toxicol Sci 2006;92:368–77.
[20] Elstner E, Müller C, Koshizuka K, Williamson EA, Park D, Asou H, et al. Ligands for peroxisome proliferator-activated receptorgamma and retinoic acid receptor inhibit growth and induce apoptosis of human breast cancer cells in vitro and in BNX mice. Proc Natl Acad Sci USA 1998;95:8806–11.

[21] Tsubouchi Y, Sano H, Kawahito Y, Mukai S, Yamada R, Kohno M, et al. Inhibition of human lung cancer cell growth by the peroxisome proliferator-activated receptor-gamma agonists through induction of apoptosis. Biochem Biophys Res Commun 2000;270(2):400–5.
[22] Muzio G, Martinasso G, Trombetta A, Di Simone D, Canuto RA, Maggiora M. HMG-CoA reductase and PPARalpha are involved in clofibrate-induced apoptosis in human keratinocytes. Apoptosis 2006;11:265–75.
[23] Tomiyama N, Yasuda N, Iwano C, Matzno S, Matsuyama K. Flow cytometric evaluation of synergistic pro-apoptotic effects of statins and clofibrates in IM-9 human lymphoblasts. Clin Exp Pharmacol Physiol 2007;34:876–80.
[24] Kim SH, Yoo CI, Kim HT, Park JY, Kwon CH, Kim YK. Activation of peroxisome proliferator-activated receptor-gamma (PPARgamma) induces cell death through MAPK-dependent mechanism in osteoblastic cells. Toxicol Appl Pharmacol 2006;215:198–207.
[25] Ryu JY, Whang J, Park H, Im JY, Kim J, Ahn MY, et al. Di(2-ethylhexyl) phthalate induces apoptosis through peroxisome proliferators-activated receptor-gamma and ERK 1/2 activation in testis of Sprague-Dawley rats. J Toxicol Environ Health A 2007;70:1296–303.
[26] Avis I, Martínez A, Tauler J, Zudaire E, Mayburd A, Abu-Ghazaleh R, et al. Inhibitors of the arachidonic acid pathway and peroxisome proliferator-activated receptor ligands have superadditive effects on lung cancer growth inhibition. Cancer Res 2005;65:4181–90.
[27] Canuto RA, Muzio G, Maggiora M, Trombetta A, Martinasso G, Autelli R, et al. Apoptosis induced by clofibrate in Yoshida AH-130 hepatoma cells: role of HMG-CoA reductase. J Lipid Res 2003;44:56–64.
[28] Barbiero G, Duranti F, Bonelli G, Amenta JS, Baccino FM. Intracellular ionic variations in the apoptotic death of L cells by inhibitors of cell cycle progression. Exp Cell Res 1995;217:410–8.
[29] Cossarizza A, Baccarani-Contri M, Kalashnikova G, Franceschi C. A new method for the cytofluorimetric analysis of mitochondrial membrane potential using the J-aggregate forming lipophilic cation 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1). Biochem Biophys Res Commun 1993;197:40–5.
[30] Szegezdi E, Logue SE, Gorman AM, Samali A. Mediators of endoplasmic reticulum stress-induced apoptosis. EMBO Rep 2006;7:880–5.
[31] Tan Y, Dourdin N, Wu C, De Veyra T, Elce JS, Greer PA. Ubiquitous calpains promote caspase-12 and JNK activation during endoplasmic reticulum stress-induced apoptosis. J Biol Chem 2006;281:16016–24.
[32] Wang KK. Calpain and caspase: can you tell the difference? Trends Neurosci 2000;23:20–6.
[33] Chawla A, Lazar MA. Peroxisome proliferator and retinoid signaling pathways co-regulate preadipocyte phenotype and survival. Proc Natl Acad Sci USA 1994;91:1786–90.
[34] Harvengt C, Desager JP. Plasma clofibric acid (CPIB) levels induced by three marketed compounds releasing clofibric acid, in volunteers. Int J Clin Pharmacol Biopharm 1977;15:1–4.
[35] MICROMEDEX® Healthcare Series, POISINDEX® Managements, Clofibrate and related agents (www.thomsonhc.com).
[36] Canuto RA, Muzio G, Bonelli G, Maggiora M, Autelli R, Barbiero G, et al. Peroxisome proliferators induce apoptosis in hepatoma cells. Cancer Detect Prev 1998;22:357–66.
[37] Matzno S, Yasuda S, Kitada Y, Akiyoshi T, Tanaka N, Juman S, et al. Clofibrate-induced apoptosis is mediated by Ca^{2+}-dependent caspase-12 activation. Life Sci 2006;78:1892–9.
[38] Nakamura M, Kuroiwa N, Kono Y, Takatsuki A. Characterization of clofibrate-induced retrograde Golgi membrane movement to the endoplasmic reticulum: clofibrate distinguishes the Golgi from the trans Golgi network. Biosci Biotechnol Biochem 2001;65:1812–23.
[39] Eizirik DL, Cardozo AK, Cnop M. The role for endoplasmic reticulum stress in diabetes mellitus. Endocr Rev 2008;29:42–61.
[40] Nakagawa T, Zhu H, Morishima N, Li E, Xu J, Yankner BA, et al. Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-beta. Nature 2000;403:98–103.
[41] Urano F, Wang X, Bertolotti A, Zhang Y, Chung P, Harding HP, et al. Coupling of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase IRE1. Science 2000;287:664–6.
[42] Oakes SA, Lin SS, Bassik MC. The control of endoplasmic reticulum-initiated apoptosis by the BCL-2 family of proteins. Curr Mol Med 2006;6:99–109.
[43] Lam M, Dubyak G, Chen L, Nuñez G, Miesfeld RL, Distelhorst CW. Evidence that BCL-2 represses apoptosis by regulating endoplasmic reticulum-associated Ca2+ fluxes. Proc Natl Acad Sci USA 1994;91:6569–73.
[44] Jiang HY, Wek RC. Phosphorylation of the alpha-subunit of the eukaryotic initiation factor-2 (eIF2alpha) reduces protein synthesis and enhances apoptosis in response to proteasome inhibition. J Biol Chem 2005;280:14189–202.
[45] Nixon RA. The calpains in aging and aging-related diseases. Ageing Res Rev 2003;2:407–18.
[46] Nakagawa T, Yuan J. Cross-talk between two cysteine protease families. Activation of caspase-12 by calpain in apoptosis. J Cell Biol 2000;150:887–94.
[47] Schotte P, Declercq W, Van Huffel S, Vandenabeele P, Beyaert R. Non-specific effects of methyl ketone peptide inhibitors of caspases. FEBS Lett 1999;442:117–21.