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ORIGINAL PAPER

Synthesized esters of ferulic acid induce release of cytochrome *c* from rat testes mitochondria

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Abstract Ferulic acid plays a chemopreventive role in cancer by inducing tumor cells apoptosis. As mitochondria play a key role in the induction of apoptosis in many cells types, here we investigate the mitochondrial permeability transition (MPT) and the release of cytochrome *c* induced by ferulic acid and its esters in rat testes mitochondria, in TM-3 and MLTC-1 cells. While ferulic acid, but not its esters, induced MPT and cytochrome *c* release in rat testes isolated mitochondria, in TM-3 cells we found that both ferulic acid and its esters induced cytochrome *c* release from mitochondria in a dose-dependent manner, suggesting a potential target of these compounds in the induction of cell apoptosis. The apoptosis induced by ferulic acid is therefore associated with the mitochondrial pathway involving cytochrome *c* release and caspase-3 activation.

Keywords Ferulic acid · Cytochrome *c* · Apoptosis · Mitochondria · TM-3 cells · MLTC-1 cells

Introduction

Apoptosis, an active and programmed form of cell death, is a multistep process (Hengartner 2000) that plays an important role in the regulation of development morphogenesis (Vaux and Korsmeyer 1999), cell homeostasis, and diseases such as cancer, stroke, and ischemic heart disease (Thompson 1995). Two apoptotic pathways by which cells can initiate and execute the cell death process, the extrinsic and the intrinsic, have been identified (Green 2000; Johnstone et al. 2002). The extrinsic pathway is initiated by ligation of transmembrane death receptors (CD95, TNF receptor, and TRAIL receptor) to activate membrane proximal caspases (caspase-8 and -10). The mammalian caspase family comprises at least 13 known members, most of which have been definitively implicated in apoptosis. *In vitro* experiments suggest that several caspases could activate by themselves, while others require activation by other caspases, acting as a proteolytic cascade (Nicholson and Thornberry 1997). Caspase-3, -6, and -7 are terminal members of caspase cascade and recognize critical cellular substrates, whose cleavage contributes to the morphological and functional changes associated with apoptosis (Thornberry and Lazebnik 1998). Caspase-3 activation also results in DNA cleavage via inactivation of an inhibitor of the DNA fragmentation factor, the endonuclease responsible for internucleosomal cleavage of chromatin (Wickremasinghe and Hoffbrand 1999). Recent findings showed that caspase-3 has a mitochondrial and cytosolic distribution in non-apoptotic cells (Mancini et al. 1998). The mitochondrial caspase-3, which is located in the intermembrane space, was shown to be activated by numerous pro-apoptotic stimuli and this activation could be blocked by bcl-2 (Mancini et al. 1998). Once the caspases are activated, the cell is irreversibly committed to cell death (Reed et al. 1997).

The intrinsic pathway is initiated in the cells by the loss of integrity of the outer mitochondrial membrane and the release of cytochrome *c* into the cytosol (Hirsch et al. 1997; Green and Reed 1998; Joza et al. 2001; Zamzami and

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Kroemer 2001). Then cytochrome *c*, an essential constituent of the respiratory chain, is released from mitochondria into the cytosol and induces a conformational change in Apaf-1 (apoptotic protease activating factor-1) that results in the activation of a cascade of caspase proteases with consequent cell death (Yang and Cortopassi 1998; Susin et al. 1999). The release of cytochrome *c* is associated with the mitochondrial permeability transition (MPT). Indeed, it is associated with depolarization of the mitochondrial inner membrane potential, loss of the H^+ gradient, uncoupling of oxidative phosphorylation, ATP depletion, mitochondrial swelling and disruption of the outer mitochondrial membrane (Wudarczyk et al. 1996; Bindoli et al. 1997; Kowaltowski et al. 1997; Bossy-Wetzel et al. 1998; Vicira et al. 2000). Among the non proteic effectors, calcium ion is the most important inducer of MPT (Petronilli et al. 1993; Schild et al. 2001).

Ferulic acid is one of the most ubiquitous compounds in nature, especially rich as an ester form in rice bran pitch, which is obtained when rice oil is produced (Yagi and Ohishi 1979). This antioxidant compound is currently expected not only to prevent lipid oxidation in food but also to prevent free-radical-induced diseases such as cancer and atherosclerosis or aging caused by oxidative tissue degeneration (Niki 1997). In general, the inhibitory effect of ferulic acid as antioxidant on lipid oxidation is due to its phenolic nucleus and its conjugated side chain forming a resonance-stabilized phenoxy radical (Frankel 2001). Although ferulic acid and its esters have been recognized as antioxidants, there are few reports on systematic evaluation of the antioxidant properties of ferulic acid and its derivatives under different conditions.

In this paper we describe the apoptotic activities of substances obtained by chemical synthesis. Our results indicate that ferulic acid, but not its synthetic esters, can induce MPT and promote cytochrome *c* release from rat testes mitochondria. In addition, a testes cell-free model of apoptosis is described consisting of a cytosolic extract from mouse Leydig cells (TM-3) and tumoral cells (MLTC-1).

Materials and methods

Chemicals

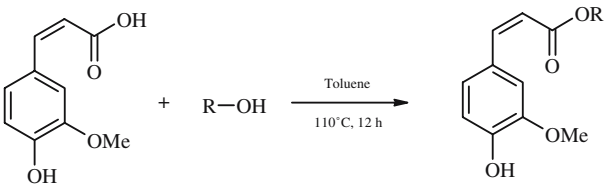
Ferulic acid was obtained from Polichimica (Bologna, Italia). Butyl alcohol, pentyl alcohol, octyl alcohol, EDTA, EGTA, SDS, acrylamide and methylenbisacrylamide were purchased from Sigma-Aldrich (Milano, Italia). DMEM/F12, horse serum, foetal calf serum, penicillin and streptomycin was from Gibco (Invitrogen Life Technologies, Italia). All other chemicals used were of the highest purity commercially available.

Synthesis of ferulate esters

The ferulate esters were synthesized according to the procedure described in the literature (EP 0681 825 B1). A solution of ferulic acid (1.0 g, 5.15 mmol) and *p*-toluensulfonic acid (0.07 g, 0.34 mmol), in 10 ml dry toluene was added to aliphatic alcohols (butyl alcohol 0.40 g, pentyl alcohol 0.48 g, and octyl alcohol 0.7 g, 5.4 mmol) under stirring and N_2 at room temperature. After addition was complete, the solution was stirred under N_2 for 12 h at 110°C. After cooling at room temperature, the solvent was evaporated under reduced pressure, and the residue was treated with 15 ml of water and the aqueous phase was extracted with chloroform (4×15 ml). The combined organic phases were collected, dried (Na_2SO_4) and the solvent was removed by rotary evaporation to give compounds 2, 3, 4 (Scheme 1) as yellow oils, and purified by Merck silica gel (60–230 mesh) column chromatography using chloroform/hexane, 60/40 (v/v), as eluent. The solvent of eluted esters was evaporated under reduced pressure. The ferulate esters were obtained with a yield of 80–90%. IR, m/z and ^{1}H-NMR data were assigned according to the literature.

Isolation of mitochondria and monitoring of the MPT

Rats were killed by decapitation, according to good practice procedures approved by the ethics committee, and testes were immediately removed. Rat testes mitochondria were isolated by differential centrifugation as described by Genchi and Olson (Genchi and Olson 2001). Mitochondria were suspended in a medium containing 250 mM sucrose, 10 mM Tris/HCl, pH 7.4, 1 mM EDTA at a concentration of 15–18 mg protein/ml. Protein concentration was determined by the Lowry procedure (Lowry et al. 1951) with BSA as the reference standard. This mitochondrial suspension either was used immediately or was frozen at −70°C. For swelling studies, mitochondria (1 mg/ml) were suspended in a solution of 250 mM sucrose, 10 mM Tris/HCl, pH 7.4,



1

2 R= $CH_3(CH_2)_3$-
3 R= $CH_3(CH_2)_4$-
4 R= $CH_3(CH_2)_7$-

Scheme 1 Reaction conditions to synthesize the compounds 2, 3 and 4

2 μM rotenone and were preincubated with 5 mM succinate. After 5 minutes, different concentrations of ferulic acid and ferulate esters or $CaCl_2$ were added and mitochondrial swelling was followed spectrophotometrically by the decrease in absorbance at 540 nm (Rigobello et al. 1999).

Release of cytochrome *c* from isolated rat testes mitochondria

1 mg of rat testes mitochondria was incubated in the presence and in the absence of ferulic acid and its esters for 1 h at 25°C in a final volume of 1 ml of buffer consisting of 250 mM sucrose, 10 mM Tris/HCl, pH 7.4. The mitochondria were separated from the supernatant by centrifugation at 13,000×g for 10 min at 4°C. The supernatant (20–30 μg of cytosolic proteins) was subjected to SDS-PAGE on 15% gel, and the proteins were transferred to a nitrocellulose membrane. This membrane was incubated at room temperature for 1 h with 5% non-fat milk in TBST (25 mM Tris–HCl pH 7.8, 150 mM NaCl, 0.1% Tween 20) and then for 2 h with a mouse monoclonal antibody to cytochrome *c* (1:1,000 dilution in TBST). After three washes with TBST the membrane was incubated for 1 h with horseradish peroxidase-conjugate antibodies to mouse immunoglobulin G (1:2,000 dilution). The membrane was washed three times more with TBST, after which immune complexes were detected with chemiluminescence reagents (ECL Amersham).

Cell cultures

The Leydig (TM-3) cell line, derived from testes of immature BALB/c mice was obtained from Dr. V. Pezzi (University of Calabria) and cultured in DMEM/F12 medium supplemented with 5% horse serum (HS) and 5% foetal calf serum (FCS), 2 mM glutamine and 1% of a stock solution containing 10,000 IU/ml penicillin and 10,000 μg/ml streptomycin. The MLTC-1 cell line, derived from testes of C57BL/6 mice, was obtained from Dr. S. Andò (University of Calabria) and cultured in DMEM/F12 medium supplemented with 10% FCS, 2 mM glutamine and 1% of a stock solution containing 10,000 IU/ml penicillin and 10,000 μg/ml streptomycin.

Cellular cultures were grown on 90 mm plastic tissue culture dishes in a humidified atmosphere of 5% CO_2 in air at 37°C. Cells from exponentially growing stock cultures were removed from the plate with trypsin (0.05% w/v) and EDTA (0.02% w/v). The trypsin/EDTA action was inhibited with an equal volume of DMEM/F12 medium. Cell number was estimated with a Burker camera and cell viability by trypan blue dye exclusion. For both cellular lines, the medium was changed twice weekly. TM-3 were subcultivated when confluent, while MLTC-1 cells were subcultured when they formed island domes.

Cytosolic extracts of TM-3 and MLTC-1 cells and detection of cytochrome *c*

Cytochrome *c* was detected by western blotting in cytoplasmatic fractions. TM-3 cells were treated with 5, 25, 50 and 100 μM ferulic acid, while MLTC-1 cells were treated with a concentration range of ferulic acid between 0.5 and 100 μM; both cellular lines were incubated for 24 h at 37°C in a humidified atmosphere of 5% CO_2 in air. In other experiments, both cells lines were treated with ferulate esters at the same concentrations and conditions. After above treatments, TM-3 and MLTC-1 cells were collected by scraping and harvested by centrifugation at 1,200×g for 10 min at 4°C. The pellets were solubilized in 36 μl lysis buffer (250 mM sucrose, 1.5 mM EGTA, 1.5 mM EDTA, 1 mM $MgCl_2$, 25 mM Tris/HCl, pH 6.8, 1 mM DTT, 10 μg/ml aprotinin, 50 mM phenylmethylsulfonylfluoride and 50 mM sodium orthovanadate). After the addition of 4 μl of 0.1% digitonine, the cells were incubated for 15 min at 4°C and centrifuged at 13,000×*g* for 30 min at 4°C. Proteins of the cytosolic fractions were determined by the Lowry method (Lowry et al. 1951). Equal amounts of protein (15–20 μg) were resolved by 15% SDS-PAGE and electrotransferred to a nitrocellulose membrane. The membrane was incubated with 5% non-fat milk in TBST over night at 4°C, followed by incubation with 1:1,000 sheep polyclonal antihuman cytochrome *c* antibody (2 h, room temperature) and then with HRP-conjugated secondary antibody (1:2,000) for 2 h at 4°C. Peroxidase activity was visualized with the Amersham Pharmacia Biotech ECL system according to the manufacturer instructions. The loading control was detected by immunoblot of β-actin protein.

Western blot analysis of caspase-3

TM-3 cells were lysed with 200 μl ice-cold PBS (phosphate buffered saline, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 136,9 mM NaCl, pH 7.2) containing 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS and protease inhibitors (1 mM phenylmethylsulfonylfluoride, 10 mg/ml aprotinin and 10 mg/ml leupeptin). Lysates were centrifuged (13,000×g at 4°C for 30 min) and the supernatant protein content was determined by the Lowry method (Lowry et al. 1951). Equal amounts of protein (20 μg) were resolved by 15% SDS-PAGE and electrotransferred to a nitrocellulose membranes. After blocking, the membranes were incubated (2 h, room temperature) with 1:2,000 rabbit polyclonal antihuman caspase-3 antibody and then (1 h, room temperature) with 1:3,000 HRP-conjugated secondary antibody. Peroxidase activity was visualized with the Amersham Pharmacia Biotech ECL system according to the manufacturer instructions. The caspase-3 protein content was determined densitometri-

cally. The loading control was detected by immunoblot of β actin protein.

Statistical analyses

Statistical differences were determined by one-way analysis of variance (ANOVA) followed by Dunnet method, and the results were expressed as mean±SD from three independent experiments. Differences were considered statistically significant for $P<0.05$.

Results

Mitochondrial swelling attributable to the MPT was monitored by measuring the decrease in optical density at 540 nm of a mitochondrial suspension (Rigobello et al. 1999). Isolated rat testes mitochondria were energized with 5 mM succinate for 5 minutes in the presence of 2 μM rotenone, after which ferulic acid and its esters (10, 50, 100 μM) or 200 μM calcium ion, a well established inducer of MPT (Petronilli et al. 1993; Schild et al. 2001), were added. Incubation of testes mitochondria with ferulic acid caused swelling in a dose-dependent manner (Fig. 1a). The presence of 200 μM calcium ion in the incubation medium together with ferulic acid synergistically stimulated the extent of swelling (Fig. 1b). On the contrary, the incubation of mitochondria with ferulate esters did not cause any swelling effect (not shown).

Mitochondria are vulnerable targets to toxic injury by a variety of compounds because of their crucial role in maintaining cellular structure and function via oxidative phosphorylation and ATP production. Mitochondrial membrane damage induces apoptosis by releasing cytochrome *c* into the cytoplasm (Hirsch et al. 1997; Green and Reed 1998; Joza et al. 2001; Zamzami and Kroemer 2001). Under normal cellular conditions, cytochrome *c* is present in the mitochondrial intermembrane space loosely bound to the inner membrane. To investigate the role of ferulic acid and its esters in inducing cytochrome *c* release, a crude preparation of rat testes mitochondria was incubated for 1 h at 25°C with increasing concentrations (5, 25, 50 and 100 μM) of ferulic acid, butyl ferulate, pentyl ferulate and octyl ferulate. After centrifugation of mitochondria, the resulting supernatants were subjected to western-blot analysis with a monoclonal antibody to cytochrome *c*. As shown in Fig. 2a, incubation of intact mitochondria with increasing concentrations of ferulic acid resulted in a increase of cytochrome *c* in the supernatants at 25, 50 and 100 μM. On the other hand, in the supernatants from mitochondria incubated in the presence of butyl ferulate, pentyl ferulate and octyl ferulate the release of cytochrome *c* was very low or not detectable (data not shown).

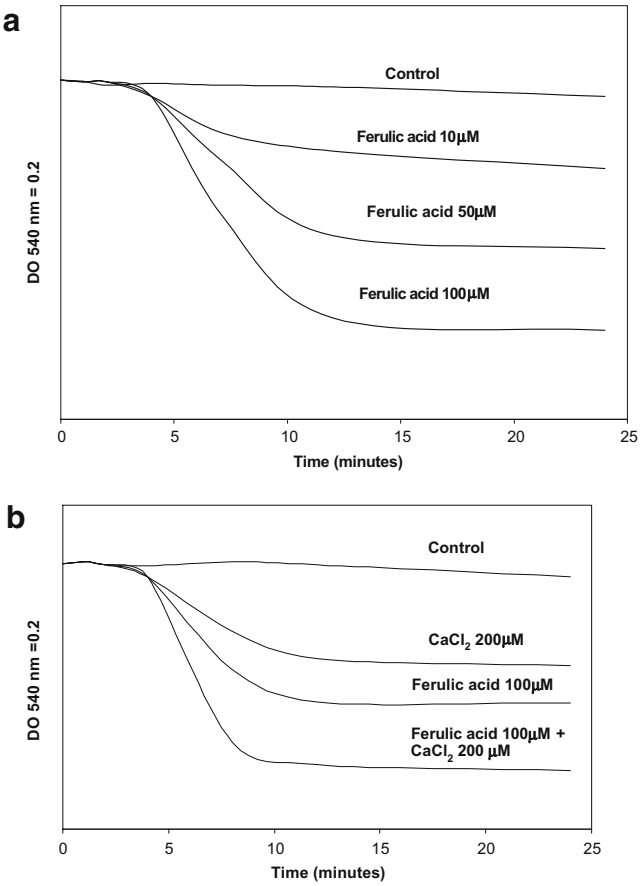


Fig. 1 Induction of MPT in rat testes mitochondria by ferulic acid and $CaCl_2$. Mitochondria were suspended in a solution of 250 mM sucrose, 10 mM TRIS/HCl, pH 7.4, in the presence of 2 μM rotenone and preincubated with 5 mM succinate for 5 min, after which (**a**) ferulic acid (10, 50 and 100 μM) and (**b**) $CaCl_2$ (200 μM), ferulic acid (100 μM) and ferulic acid (100 μM) plus $CaCl_2$ (200 μM) were added. The mitochondrial swelling was monitored by measuring the decrease in optical density at 540 nm

To establish a testes cell-free model of apoptosis, we obtained cytosolic extracts from TM-3 and MLTC-1 cells. Apoptosis is an orderly cascade of cellular events that ultimately results in the demise of the cell. Cytochrome c release from mitochondria has also been shown to be a central event in the regulation of apoptosis. To examine whether ferulic acid and its esters induce release of cytochrome *c* in TM-3, the cells were treated without (only vehicle) and with 5, 25, 50 and 100 μM ferulic acid. After 24 h exposure, TM-3 cells were collected, lysed and the cytosolic protein fractions were assayed for cytochrome *c* to determine its presence into the cytoplasm. As shown in Fig. 2b, the amount of cytochrome *c* released from the cells treated with 50 μM ferulic acid was higher than control ($P<0.05$). The release of cytochrome *c* was evident only after 24 h incubation (data not shown).

During the process of apoptosis, the cytochrome *c*, released into the cytosol from the mitochondria, activates the caspases, a family of killer proteases. In particular, the

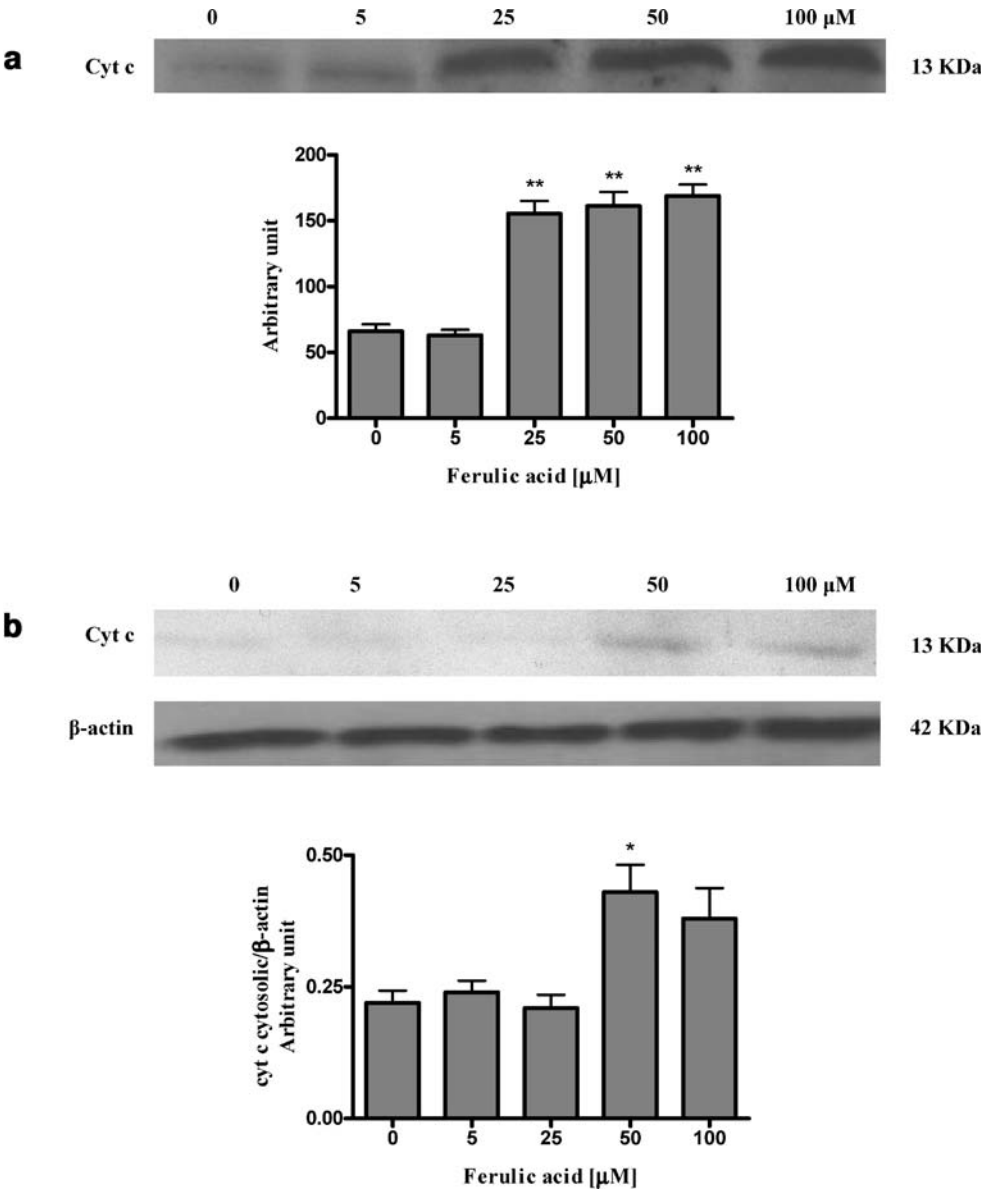


Fig. 2 Effect of ferulic acid on cytochrome *c* release from rat testes isolated mitochondria and TM-3 cells. Mitochondria were incubated with ferulic acid at 0, 5, 25, 50 and 100 μM for 1 h (**a**), TM-3 cells were incubated with ferulic acid at 0, 5, 25, 50 and 100 μM for 24 h (**b**) and cytochrome *c* release was measured by immunoblotting (representative of three experiments) The cytochrome *c* protein content was determined densitometrically. β-Actin, used as internal control, was detected at the position corresponding to a molecular weight of 42 KDa. Results are presented as the mean±SD. **P*<0.05 compared to the control; ***P*<0.01 compared to the control

activated caspase-3 has many cellular targets, and produces the morphological features of apoptosis. Western blot analyses, using a caspase-3 antibody that recognizes the caspase-3 holoenzyme as well as the p17 cleavage product of caspase-3, were performed to investigate whether enzymatic processing occurred following ferulic acid exposure. At 24 h post-exposure, 25, 50 and 100 μM ferulic acid treated cells showed a decrease of the 32 kDa caspase-3 protein, as well as an increase of the 17 kDa cleavage product (Fig. 3).

Release of cytochrome *c* by ferulic acid esters

To compare the efficacy of ferulic acid with pharmaceutically active compounds, we have evaluated the release of cytochrome *c* by esters of ferulic acid obtained by chemical synthesis. This could be of relevance, offering not only a different therapeutical efficacy but a distinct tissue distribution and pharmacokinetics, thus offering different side effects.

Therefore we have treated TM-3 cells with 5, 25, 50 and 100 μM of butyl ferulate, pentyl ferulate and octyl ferulate. Greater cytochrome *c* release has been found when treating TM-3 cells with 50 μM of each ester. Octyl ferulate, among the esters used, is the most effective in inducing cytochrome *c* release (Fig. 4).

To verify that ferulic acid and its esters act as apoptotic inducers, we have treated tumoral Leydig cells at concentrations lower in a range among 0.5–100 μM than those used in TM-3 cells. After 24 h exposure, MLTC-1 cells were collected, lysed and the cytosolic protein fractions

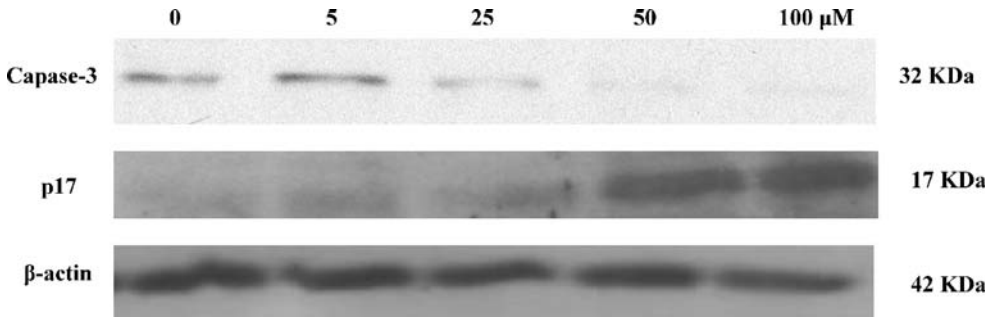


Fig. 3 Effect of ferulic acid on caspase-3 and p17 fragment activation in TM-3 cells. Cells were incubated with ferulic acid at 0, 5, 25, 50 and 100 μM. At 24 h post-exposure, cells were washed with PBS. Equal amounts of cytosolic proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and probed as described in Materials and methods. Caspase-3 and p17 fragment were detected by chemiluminescence. β-Actin, used as internal control, was detected at the position corresponding to a molecular weight of 42 KDa

were assayed for cytochrome *c* to determine its presence into the cytoplasm. As shown in Fig. 5a, the amount of cytochrome *c* released from the cells treated with ferulic acid was highly significant ($P<0.01$) already at 0.5 μM. However, as regards ferulate esters, the release of cytochrome *c* into the cytoplasm was significant ($P<0.05$) at 25 μM for both butyl and octyl ferulate and highly significant ($P<0.01$) for pentyl ferulate at the same concentration (Fig. 5b–d).

Discussion

Current approaches to cancer treatment are mostly based on cytotoxic and cytostatic mechanisms to eliminate malignant cells. These pharmacological strategies, although efficacious toward the malignant cells, show a number of toxic side effects which frequently hamper or drastically reduce their use. A newer dimension in cancer management is the increasing awareness that chemoprevention, namely the

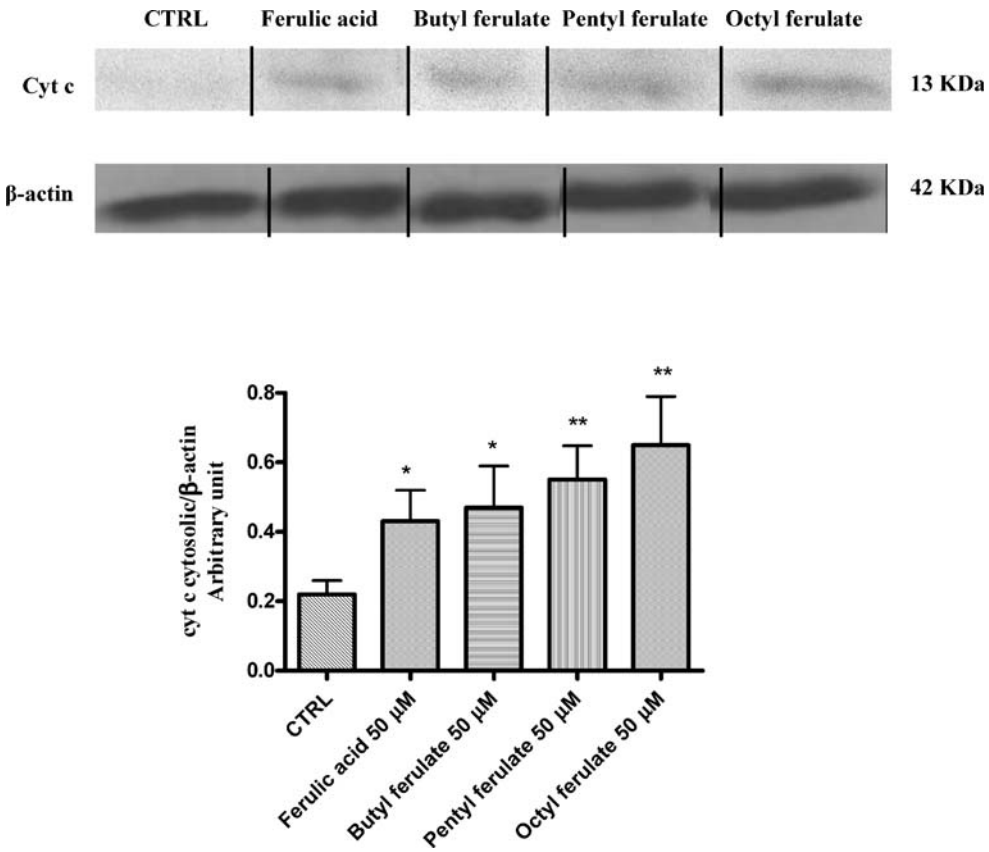


Fig. 4 Effect of ferulic acid and its esters on cytochrome *c* release from TM-3 cells. TM-3 cells were incubated in the presence of free-medium, ferulic acid, butyl ferulate, pentyl ferulate and octyl ferulate at 50 μM. At 24 h post-exposure, cells were washed with PBS; equal amounts of cytosolic proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and probed as described in Materials and methods. Cytochrome *c* was detected by chemiluminescence. The cytochrome *c* protein content was determined densitometrically. β-Actin, used as internal control, was detected at the position corresponding to a molecular weight of 42 KDa. Results are presented as the mean±SD. *$P<0.05$ compared to the control; **$P<0.01$ compared to the control

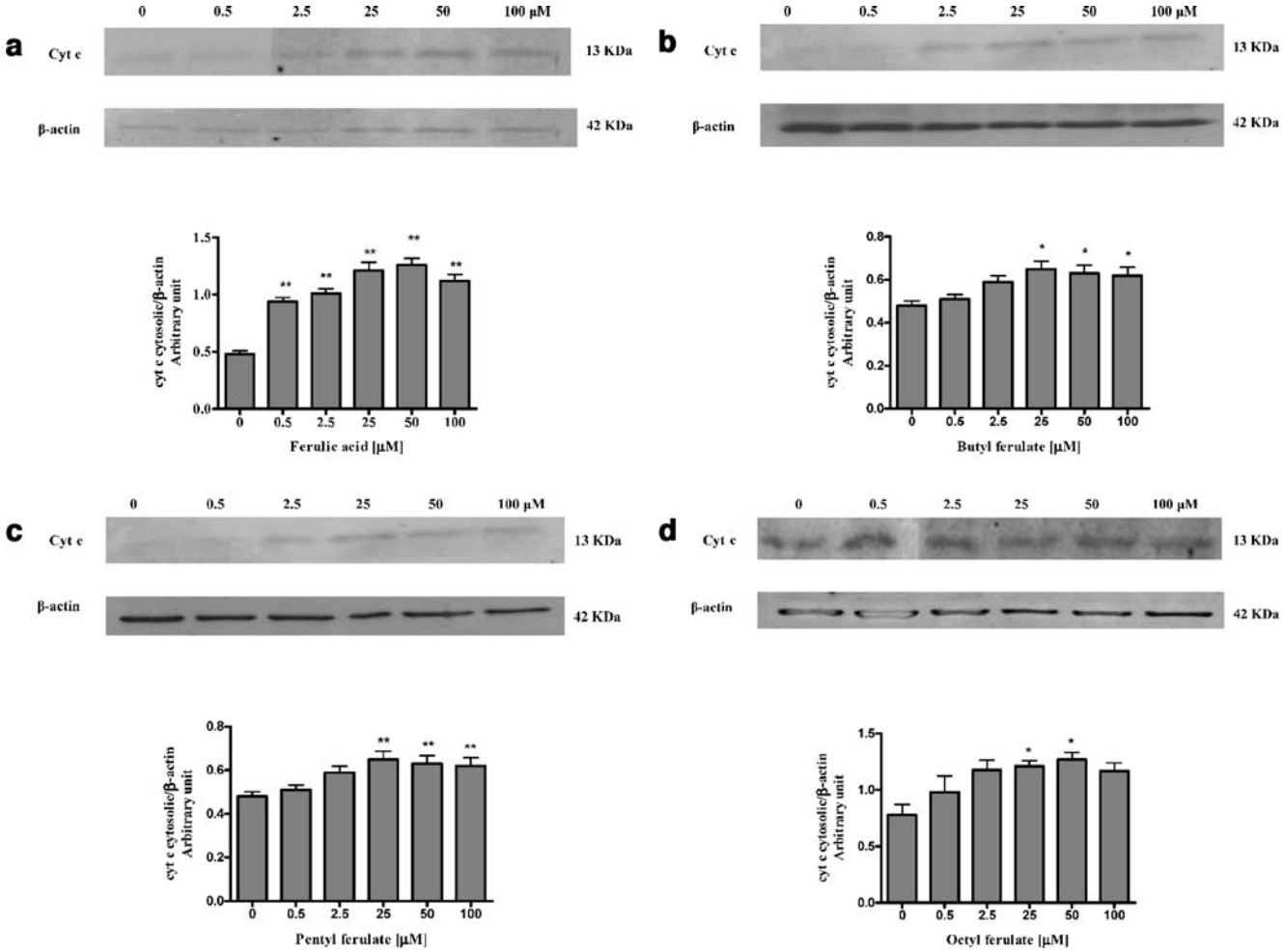


Fig. 5 Effect of ferulic acid and its esters on cytochrome *c* release from MLT-1 cells. Cells were incubated with ferulic acid (**a**), butyl ferulate (**b**), pentyl ferulate (**c**) and octyl ferulate (**d**) at 0, 0.5, 2.5, 25, 50 and 100 μM for 24 h and cytochrome *c* release was measured by immunoblotting (representative of three experiments). The cytochrome *c* protein content was determined densitometrically. β-Actin, used as internal control, was detected at the position corresponding to a molecular weight of 42 KDa. Results are presented as the mean±SD. **P*<0.05 compared to the control; ***P*<0.01 compared to the control

administration of chemical agents (both natural and synthetic) to prevent the early events of carcinogenesis, could be the most direct way to counteract malignancy development and progression (Della Ragione et al. 2000). In the search for new cancer chemopreventive compounds, hundreds of naturally occurring molecules have been evaluated over the past few years. Among the agents able to lower the rate of malignant transformation, antioxidants appear to be very promising. Indeed, diets rich in antioxidant molecules are clearly associated with a diminished risk of cancer at various anatomical sites (Della Ragione et al. 2000). However, some intervention studies have questioned the effectiveness of specific antioxidants in tumor prevention. This suggests that the use of these compounds as chemopreventive agents must await more detailed knowledge of their mechanism of action and their interactions with genetic phenotypes and environment (Della Ragione et al. 2000).

Numerous studies have suggested that ferulic acid and its related compounds exert antioxidants activities (Yagi and Ohishi 1979) and they are currently expected not only to prevent lipid oxidation in food but also to prevent free-radical-induced diseases such as cancer and atherosclerosis or aging caused by oxidative tissue degeneration (Niki 1997). Apoptosis is an active mode of programmed cell death that is induced by a variety of physiological and pathological stimuli. However, apoptosis can also be induced by a variety of toxicants, including many toxic organic compounds, resulting in loss of cell viability. The translocation of cytochrome *c* into the cytosol represents a central point in many forms of apoptosis, since cytochrome *c* is a fundamental component of the apoptosome, responsible for caspase activation. In the current report we have described a novel and unexpected effect of ferulic acid: the induction of cytochrome *c* release from rat testes mitochondria.

We have shown that ferulic acid but not its esters induce MPT opening in isolated rat testes mitochondria and that calcium added in the incubation buffer increases this swelling, resulting in release of cytochrome *c* into the

cytoplasm. On the other hand, in TM-3 cells we have found that ferulic acid and its esters induce apoptosis, associated primarily with the mitochondrial pathway involving release of cytochrome *c*. Ferulic acid, in fact, induces release of cytochrome *c* after 24 h of incubation. The release of cytochrome *c* may result in the activation of members of a family of serine-proteases such as the caspase, another hallmark of apoptosis. Caspases play a central role in terminal biochemical events that ultimately lead to apoptotic cell death. Western blot analysis also indicates marginal appearance of the 32 kDa and 17 kDa caspase-related proteins in TM-3 cells treated with ferulic acid.

As regards ferulate esters, butyl ferulate and pentyl ferulate were less effective than octyl ferulate and the release of cytochrome *c* was clearly evident when their concentrations were 50 μM. The same experimental procedures were done with tumoral Leydig MLTC-1 cells at concentrations lower than those used in TM-3 cells. After 24 h exposure with ferulic acid cytochrome *c* was found into the cytoplasm already at 0.5 μM ferulic acid. The same result is not obtained with ferulate esters; the release began to be evident at 25 μM.

Taken together these observations suggest that ferulic acid activates the mitochondrial pathway of the apoptotic process inducing cytochrome *c* release in isolated mitochondria and in Leydig cells both normal and tumoral, while its esters are able to produce this results only in TM-3 and in MLTC-1 cells, but not in isolated mitochondria. Probably the MPT and the release of cytochrome *c* induced by ferulic acid in isolated mitochondria is due to its phenolic nucleus and its conjugated side chain. In case of complex biological system both ferulic acid and its esters provoked release of cytochrome *c* from mitochondria but there was no significant difference among the release induced by ferulic acid and its esters both in TM-3 and in MLTC-1 cells. So the active compound should be ferulic acid and not the long chain of the esters; in biological systems in fact there are enzymes (esterases) that can hydrolize ester bonds.

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