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## Research Article

# Increased mitochondrial palmitoylcarnitine/carnitine countertransport by flavone causes oxidative stress and apoptosis in colon cancer cells

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**Abstract.** Cancer cell metabolism is characterized by limited oxidative phosphorylation in order to minimize oxidative stress. We have previously shown that the flavonoid flavone in HT-29 colon cancer cells increases the uptake of pyruvate or lactate into mitochondria, which is followed by an increase in $O_2^{-\cdot}$ production that finally leads to apoptosis. Similarly, a supply of palmitoylcarnitine in combination with carnitine induces apoptosis in HT-29 cells by increasing the mitochondrial respiration rate. Here we show that flavone-induced apoptosis is increased more than twofold in the presence of palmitoylcarnitine due to increased mitochondrial fatty acid transport and the subsequent metabolic generation of $O_2^{-\cdot}$ in mitochondria is the initiating factor for the execution of apoptosis.

**Key words.** HT-29 human colon cancer cells; superoxide anion generation; mitochondrial apoptosis pathway; fatty acid transport.

Various metabolic changes have been observed to occur in oncogenesis that serve to accomplish the special metabolic requirements of cancer cells. These changes include a high rate of glycolysis associated with an increased rate of glucose transport, reduced pyruvate oxidation with increased production of lactate, decreased glycerol-3-phosphate shuttle and malate-aspartate shuttle activities, increased glycerol and fatty acid turnover, and a reduced fatty acid oxidation rate [for a review, see 1]. The low rate of oxidative phosphorylation in cancer cells is thought to provide protection from reactive oxygen species (ROS)-mediated cellular damage during phases of DNA replication and high biosynthetic load [2]. We previously demonstrated that an increase in the rate of apoptosis in HT-29 human colon cancer cells is closely associated with an accelerated mitochondrial $O_2^{-\cdot}$ generation rate when lactate or pyruvate uptake into mitochondria is increased by flavone and, thereby, substrate availability for oxidative metabolism is enhanced [3]. Similarly, when palmitoylcarnitine in the presence of carnitine is provided to HT-29 cells, an increased generation of $O_2^{-\cdot}$ inside mitochondria is observed that promotes apoptosis initiation [4]. Carnitine here was proven to be a limiting factor for fatty acid import into mitochondria in colon cancer cells [1, 5] with no apoptosis observed when only palmitoylcarnitine was provided to the cells [4].

In the present study, we investigated whether flavone, beside increasing mitochondrial pyruvate or lactate transport, can increase fatty acid import into

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mitochondria as a fuel for mitochondrial beta-oxidation and respiration. Confocal laser scanning microscopy (CLSM) was used to assess mitochondrial uptake of a fluorescent palmitic acid analogue and to determine the role of $O_2^{-\cdot}$ in the apoptotic response of the cells. Caspase activation and nuclear fragmentation served as early and late apoptosis markers, respectively.

## Materials and methods

**Materials.** Flavone and Hoechst 33258 were purchased from Sigma (Deisenhofen, Germany). Media and supplements for cell culture were obtained from Invitrogen (Karlsruhe, Germany). Cell culture plates were from Renner (Dannstadt, Germany) and Quadriperm wells were obtained from Merck (Darmstadt, Germany). Proxylfluorescamine, MitoTracker Red CMXRos and 16-(9-anthroyloxy)-palmitic acid were from Bioprobes (Leiden, The Netherlands) and the fluorogenic caspase-3 substrate acetyl-aspartyl-glutamyl-valyl-aspartyl-amino-4-methyl-coumarine (Ac-DEVD-AMC) was obtained from Calbiochem (Bad Soden, Germany). The UV test for determining carnitine levels was obtained from Boehringer (Mannheim, Germany).

**Cell culture.** HT-29 cells (passage 106) were provided by the American Type Culture Collection (Rockville, MD, USA.) and used between passage 150 and 200. Cells were cultured and passaged in RPMI-1640 supplemented with 10% FCS and 2 mM glutamine. Antibiotics added to the medium were 100 U/ml penicillin and 100 µg/ml streptomycin. The cultures were maintained in a humidified atmosphere of 95% air and 5% $CO_2$ at 37 °C. Cells were passaged at preconfluent densities using of a solution containing 0.05% trypsin and 0.5 mM EDTA.

**Detection of apoptosis.** Caspase-3-like activity was measured as described previously [6] based on the method of Nicholson et al. [7]. In brief, HT-29 cells were seeded at a density of $5 \times 10^5$ per well onto 6-well plates and allowed to adhere for 24 h. Cells were then exposed for the times indicated in the figures and figure legends to the test compounds. Subsequently, cells were trypsinized, cell numbers were determined and then cells were centrifuged at 2500 g for 10 min. Cytosolic extracts were prepared by adding 750 µl of a buffer containing 2 mM EDTA, 0.1% CHAPS, 5 mM DTT, 1 mM PMSF, 10 µg/ml pepstatin A, 20 µg/ml leupeptin, 10 µg/ml aprotinin and 10 mM HEPES/KOH, pH 7.4, to each pellet and homogenizing by ten strokes. The homogenate was centrifuged at 100,000 × g at 4 °C for 30 min and the cytosolic supernatant was incubated with the fluorogenic caspase-3 tetrapeptide-substrate Ac-DEVD-AMC at a final concentration of 20 µM. Cleavage of the caspase-3 substrate was followed by determination of emission at 460 nm after excitation at 390 nm using a fluorescence microtiter plate reader (Fluoroskan Ascent; Thermo Electron Corporation, Dreieich, Germany).

Nuclear fragmentation as a late marker of apoptosis was determined by staining DNA with Hoechst 33258. HT-29 cells ($3 \times 10^4$) were grown on glass slides placed into Quadriperm wells and then incubated with the test compounds for 36 h. Thereafter, cells were washed with PBS, allowed to air-dry for 30 min and then fixed with 2% paraformaldehyde prior to staining with 1 µg/ml Hoechst 33258 and visualization under an inverted fluorescence microscope (Leica DMIRBE, Bensheim, Germany). Photographs were taken from at least three independent cell batches and apoptotic cells were determined according to the number of cells displaying chromatin condensation and nuclear fragmentation versus total cell counts.

**Confocal laser scanning microscopy.** CLSM (TCS SP2 microscope; Leica) was used for quantification of mitochondrial uptake of the fluorescent 16-(9-anthroyloxy) analogue of palmitic acid and mitochondrial $O_2^{-\cdot}$ generation. For staining of mitochondria, cells were grown on glass slides placed into Quadriperm wells and loaded with 500 nM MitoTracker Red CMXRos for the last 30 min of incubation. For detection of 16-(9-anthroyloxy)-palmitic acid uptake into mitochondria, cells were incubated with 100 µM of the fatty acid analogue for 4 h. For detection of mitochondrial $O_2^{-\cdot}$, cells were loaded with 50 µM of 5-(2-carboxyphenyl)-5-hydroxy-1-((2,2,5,5-tetramethyl-1-oxypyrrolidin-3-yl) methyl)-3-phenyl-2-pyrrolin-4-one (proxylfluorescamine) for the last 2 h. Cysteine (200 µM) was added to the incubation medium to yield an increase in the emission of proxylfluorescamine fluorescence based on the reduction of the fluorophore nitroxide to its corresponding hydroxylamine in the presence of superoxide [8]. Fluorescence of 16-(9-anthroyloxy)-palmitic acid and proxylfluorescamine was detected after excitation with the UV-laser at emissions of 440–480 nm and fluorescence of MitoTracker Red CMXRos was detected after excitation at 543 nm at emisions of 590–650 nm. The fluorescence ratios of 16-(9-anthroyloxy)-palmitic acid and proxylfluorescamine over MitoTracker were determined for the mitochondrial areas only using the Leica Confocal Software, Version 2.5.

**Determination of free carnitine.** Carnitine levels were determined separately in a cytosolic and a mitochondria-enriched fraction prepared from a homogenate of HT-29 cells. HT-29 cells exposed to the test compounds in 75-cm$^2$ flasks were harvested into a 250 mM sucrose solution. Cells were pelleted subsequently by centrifugation for 10 min at 2500 g. The pelleted cells were permeabilized through a syringe with a 24-G needle using 0.025% digitonin in a buffer containing 250 mM sucrose, 2.5 mM $MgCl_2$, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, complete mini protease inhibitor cocktail, and 20 mM Hepes/Tris, pH 7.2 [9]. To allow 95–100% of the cells to be permeabilized, the cells were incubated for 10 min on ice by gentle agitation and completeness of permeabilization was assessed by Trypan Blue exclusion. Separation of organelles and cytosol was achieved by centrifugation at 13,000 g for 2 min at 4 °C. The supernatant (cytosol) was carefully removed and the pellet, containing mitochondria, was solubilized in a buffer containing 150 mM NaCl, 1% Triton-X-100, 0.5% deoxycholic acid, 0.1% SDS, protease inhibitor cocktail, and 50 mM Tris-HCl, pH 8.0 [9]. Protein content was determined by the Bradford reaction. Carnitine levels in the cytosol and mitochondrial fraction from HT-29 cells were determined according to the manufacturer´s instructions using a UV test. Absorbance of NADH was measured using a multiwell plate reader (Multiskan Ascent; Thermo Electron Corporation).

**Calculations and statistics.** Variance analysis between groups was performed by one-way ANOVA and significance of differences between groups was determined by a Student's t-test (GraphPadPrism, San Diego, Calif.). For each variable at least three independent experiments were carried out. Data are given as the mean ± SE.

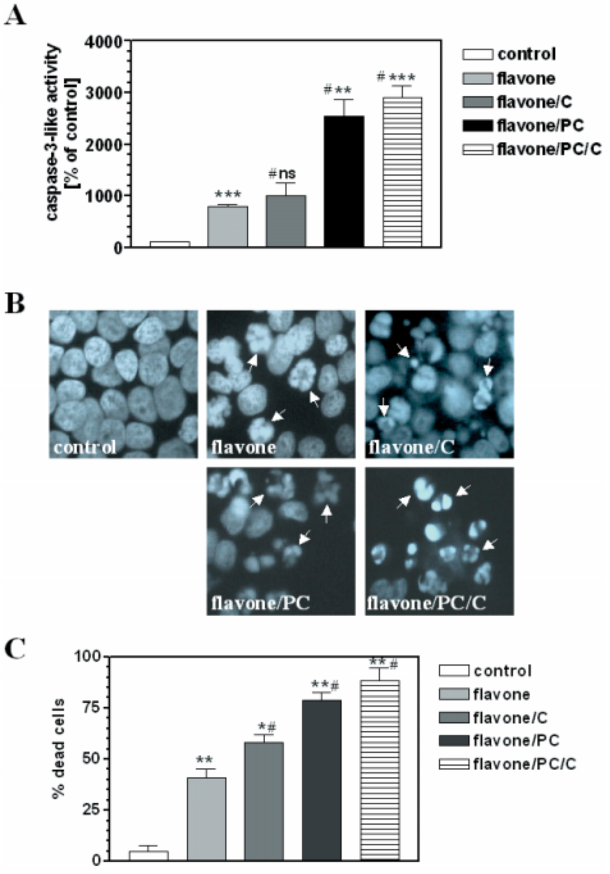

Figure 1. Flavone-induced apoptosis is potently enhanced by palmitoylcarnitine. (*A*) Caspase-3-like activity was assessed in HT-29 cells incubated for 24 h with medium alone (control) or with 150 μM flavone in the absence or presence of either 2 mM carnitine (C), 100 μM palmitoylcarnitine (PC), or a combination of PC and C, by determining the cleavage rate of Ac-DEVD-AMC. Caspase-3-like activity of cells treated with medium alone was set as 100%. $^{***}p < 0.001$ versus control; $^{\#}$ indicates comparisons versus flavone-treated cells: $^{ns}$ p not significantly different, $^{**}p < 0.01$, $^{***}p < 0.001$. (*B*) Effects of treatments on nuclear fragmentation (arrows) were assessed after 36 h by Hoechst 33258 staining in HT-29 cells. (*C*) Percentage of cells displaying signs of chromatin condensation and DNA fragmentation as shown in *B* after treatment of cells as given in *A*. $^{**}p < 0.01$ versus control; $^{\#}$ indicates comparisons versus flavone-treated cells; $^{*}p < 0.05$, $^{**}p < 0.01$.

## Results

Flavone-induced apoptosis is potently increased by palmitoylcarnitine. We have previously shown that exposure of HT-29 cells to 150 μM flavone for 24 h leads to a 8-fold increase in caspase-3-like activity as compared to control cells [6]. A similar activation of caspase-3 was achieved when cells were treated with 100 μM palmitoylcarnitine but only when simultaneously 2 mM carnitine was provided [4]. Both, palmitoylcarnitine and carnitine when given alone failed to increase caspase-3-like activity [4]. Here, we demonstrate that the flavone-induced activation of caspase-3-like activity was not significantly increased by providing extra carnitine to cells (fig. 1A). However, when palmitoylcarnitine was supplied in addition to flavone, a further increase in caspase-3-like activity was observed that resulted in a 25-fold higher activity than found in control cells (fig. 1A). Maximal caspase-3-like activities were achieved by the combination of flavone, palmitoylcarnitine and carnitine (fig. 1A). Increased caspase-3-like activities always resulted in concomitant DNA fragmentation rates, with 40% of the cells displaying fragmentation when flavone was applied alone, 60% when flavone and carnitine were provided, 80% when flavone and palmitoylcarnitine were co-administered, and 90% of cells in the presence of flavone, palmitoylcarnitine, and carnitine (figs. 1B, C). Less than 5% of control cells showed DNA fragmentation (figs. 1B, C).

**Flavone enables mitochondrial uptake of palmitoylcarnitine in HT-29 cells by increasing the levels of free carnitine in mitochondria.** Intrinsic uptake of the fluorescent palmitic acid analogue 16-(9-anthroyloxy)-palmitic acid into mitochondria of HT-29 cells is generally low but can be increased by the provision of free carnitine, as shown previously [4]. Here, we found that flavone enhanced mitochondrial uptake of

16-(9-anthroyloxy)-palmitic acid significantly ($p < 0.05$) (fig. 2A). The combination of flavone and carnitine did not significantly enhance fatty acid uptake into mitochondria further than observed for either flavone or carnitine alone (fig. 2A). When carnitine was determined in cells exposed to flavone, the mitochondria-enriched cell fraction showed drastically increased free carnitine levels, whereas carnitine levels in the cytosolic fraction remained unaffected by the treatment (fig. 2B).

**Superoxide anions ($O_2^{-}$) mediate the effects of flavone and palmitoylcarnitine on apoptosis.** Flavone was previously shown to induce the generation of mitochondrial $O_2^{-}$ in HT-29 cells and scavenging of $O_2^{-}$ blocked flavone-induced apoptosis [10]. We also showed that a similar mechanism promotes apoptosis by the combination of palmitoylcarnitine and carnitine and that quenching mitochondrial $O_2^{-}$ prevented apoptosis execution [4]. Here we demonstrate that a combination of flavone and palmitoylcarnitine is not only associated with an increased mitochondrial fatty acid uptake but also leads to a pronounced production of $O_2^{-}$ inside mitochondria (fig. 3A). The slight increase in $O_2^{-}$ in the cytosol in cells exposed to flavone and palmitoylcarnitine (fig. 3A) is most likely the consequence of diffusion of $O_2^{-}$ [11, 12] generating a gradient in the vicinity of the mitochondria. When $O_2^{-}$ was quenched using the tissue-permeable benzoquinone [13, 14] in the presence of flavone and palmitoylcarnitine (fig. 3A), activation of caspase-3 (fig. 3B) as well as nuclear fragmentation (fig. 3C) were markedly reduced.

## Discussion

Most if not all characteristic features of tumor cell metabolism are an adaptation to the special local environment [15, 16]. These changes allow tumor cells

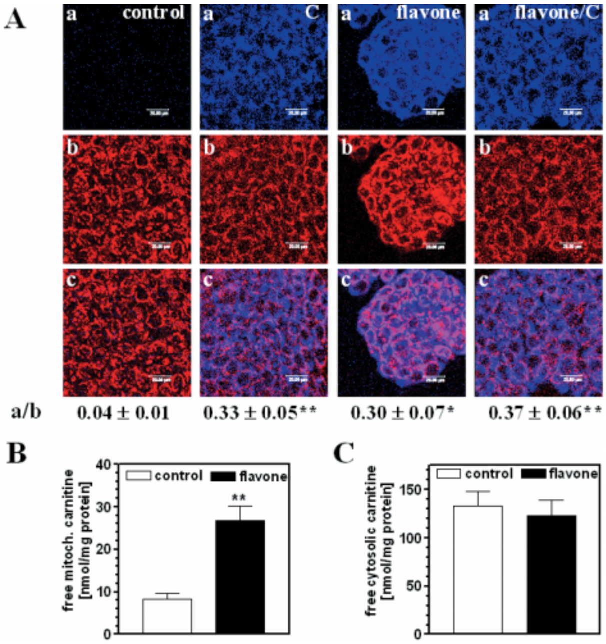

Figure 2. Flavone enhances mitochondrial uptake of a fluorescent palmitic acid analogue into mitochondria of HT-29 cells through increased levels of free carnitine. (*A*) Cells were treated for 4 h with 100 µM 16-(9-anthroyloxy)-palmitic acid either in medium alone (control) or in the presence of 2 mM carnitine (C), or 150 µM flavone, or 150 µM flavone plus 2 mM carnitine. Mitochondria of cells were stained by incubating the cells with MitoTracker for the last 30 min of incubation. The fluorescence ratios of 16-(9-anthroyloxy)-palmitic acid (a) over MitoTracker (b) were determined for the mitochondrial areas only, using CLSM. The overlay of a and b is shown in c. *$p < 0.05$ versus control; **$p < 0.01$ versus control. Levels of free carnitine in mitochondria (*B*) and the cytosol (*C*) of HT-29 cells incubated for 2 h in the absence (control) or presence of 150 µM flavone were determined UV-spectroscopically. **$p < 0.01$ versus control.

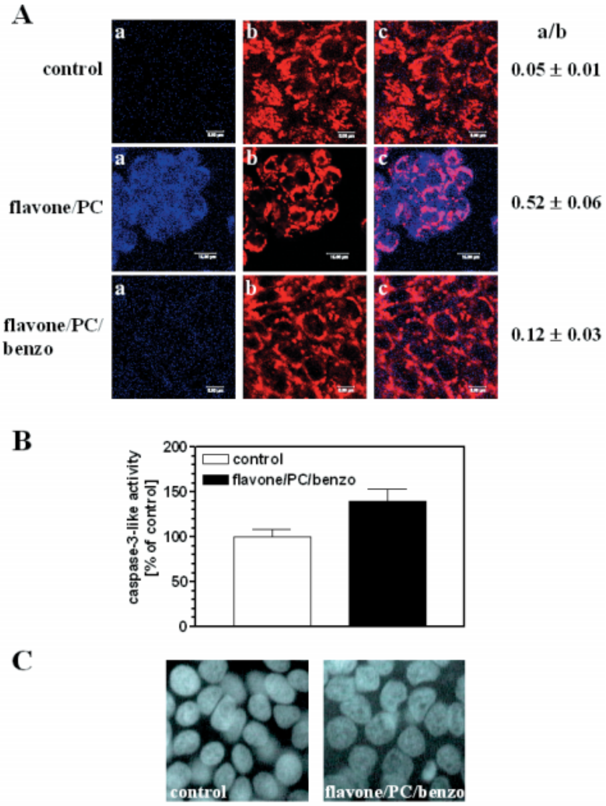

Figure 3. Flavone/palmitoylcarnitine-triggered apoptosis is mediated by mitochondrial $O_2^{-}$. (*A*) Cells were exposed to medium alone (control), or to 150 µM flavone/2 mM palmitoylcarnitine (PC) with or without 10 µM benzoquinone (benzo) for 6 h. Cells were loaded with proxylfluorescamine for the detection of $O_2^{-}$ (a) in combination with MitoTracker for the visualisation of mitochondria (b). The fluorescence ratios of a over b were determined for the mitochondrial areas only. The overlay of a and b is displayed in c. (*B*) Caspase-3-like activities were determined in HT-29 cells incubated as indicated in (A) for 24 h. (*C*) Nuclear fragmentation was not observed in control cells or in cells treated with flavone/PC/benzo as assessed by Hoechst 33258 staining.

to survive and invade [17] even under hypoxic conditions [18] and the adaptation to a low oxygen tension becomes a crucial step in tumor progression. Associated with the metabolic changes is a resistance toward death signals as a key mechanism allowing tumor development [19]. The central importance of tumor-specific metabolic alterations is further stressed by the fact that some of the genetic alterations that directly promote tumor cell growth affect enzymes of metabolic pathways [20, 21]. The anaerobic use of glucose as an energy source through glycolysis by the so-called 'Warburg effect' is a common feature of most tumors [22]. Moreover, cancer cell metabolism is also characterized by a low rate of fatty acid oxidation [22–24]. Although energy yield is low when substrates are not completely oxidized, these adaptations in rapidly growing cancer cells at the same time minimize ROS production and prevent DNA and proteins from being damaged by oxygen radicals when produced during oxidative phosphorylation [2]. In HT-29 human colon cancer cells, an impaired transport of the glycolytic end products pyruvate and/or lactate into mitochondria prevents substrate oxidation [3]. Exposing these cells to flavone increased mitochondrial pyruvate/lactate uptake with a concomitant increase in ATP levels [25], but also an increased generation of mitochondrial $O_2^{-\cdot}$ followed by the occurrence of apoptosis [3]. When, similarly, mitochondrial fatty acid import and oxidation were increased, HT-29 cells again showed a substantial increase in production of mitochondrial $O_2^{-\cdot}$ and apoptosis [4]. Here, we demonstrate that exposure of HT-29 cells to flavone causes an increase in mitochondrial levels of free carnitine that, in turn, promotes mitochondrial uptake of palmitoylcarnitine and oxidation. Our data suggest that flavone not only activates monocarboxylate transporters of the mitochondrial membrane [3] but also transporters for free carnitine, such as organic cation transporters [26], although their membrane localization and regulation are still unknown. Interestingly, the levels of free carnitine in mitochondria were increased in HT-29 cells by flavone to levels found in the non-transformed colonic epithelial cell line NCOL-1, whereas cytosolic levels of free carnitine were essentially the same in both cell lines (data not shown). Together with the finding that mitochondrial uptake of long-chain fatty acids in NCOL-1 cells is not dependent on exogenous carnitine supply [4], the data in HT-29 cells strongly suggest that the enhanced mitochondrial carnitine uptake that allows then an increased countertransport with acylcarnitines via the acylcarnitine/carnitine-translocase is the rate-limiting step for beta-oxidation of fatty acids in colon cancer cells but not in non-transformed cells. In both cell lines, oxidation of fatty acids inevitably leads to the generation of mitochondrial $O_2^{-\cdot}$. In NCOL-1 cells, however, this can be compensated due to their higher antioxidative capacity [27]. When the antioxidative capacity is reduced, high substrate oxidation is accompanied by apoptosis induction also in NCOL-1 cells [27].

Beside accelerating mitochondrial fatty acid import, flavone may also promote release of free fatty acids from endogenous triglycerides, as suggested by the apoptosis-enhancing effects of carnitine in flavone-treated cells. However, provision of external fatty acids (i.e. palmitoylcarnitine) is much more effective in apoptosis induction and execution in flavone-exposed cells than providing carnitine. This suggests that the availability of free fatty acids for beta-oxidation is similarly limiting acetyl-CoA generation for oxidative metabolism.

In conclusion, our studies provide evidence that a combination of flavone and palmitoylcarnitine enables an efficient delivery of substrates to mitochondria for oxidation in HT-29 human colon cancer cells that normally utilize mainly glucose via glycolysis. Reversing the metabolic phenotype of transformed colonocytes toward that of a normal cell is associated with a markedly enhanced generation of mitochondrial $O_2^{-\cdot}$. Transformed colonocytes appear to be particularly vulnerable to an increased mitochondrial $O_2^{-\cdot}$ load and respond rapidly with an induction of the apoptosis program leading to a "metabolic suicide".

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